

UNIVERSITE DE GENEVE

FACULTE DES SCIENCES

Département de biochimie

Laboratoire de chimie biologique
et organique spéciale

M. J. DESHUSSES

Professeur-assistant

Studies on the mechanism of action of 2-C-methylene-myo-inositol oxide

THESE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de docteur ès sciences, mention chimique

par

Pukh Raj SHARMA

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Thèse N° 1654

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La Faculté des sciences, sur le préavis de M. J. Deshusses, professeur-assistant, directeur de thèse et d'une Commission composée des professeurs U. Leupold (Université de Berne), Th. Posternak (Faculté des sciences) et G. Turian (Faculté des sciences), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 1er avril 1974

Le doyen :
Jean Muller

Thèse No 1654



I wish to express my gratitude to Professor Th. Posternak for providing all facilities for this work in his laboratory, for his constant encouragement and interest in the study and for financial assistance.

It is also a great pleasure to express my warmest thanks to Professor J. Deshusses, the director of this thesis and my friend, for not only guiding me throughout the course of this study with utmost patience and stimulating interest, but also for making my stay in Geneva a very pleasant experience.

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The financial support from Volkart Foundation is gratefully acknowledged.

*Dedicated to my MOTHER
and
as a homage to my late FATHER*

(How shall we extol thee, who are born of thee? — Benson)

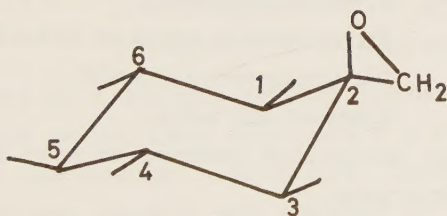
STUDIES

ON

THE MECHANISM OF ACTION

OF

2-C-METHYLENE-MYO-INOSITOL OXIDE



2-C-Methylene-myo-inositol oxide
(myo-epoxide)

ABBREVIATIONS

MI	<u>Myo</u> -inositol
MO,OX	2-C-Methylene- <u>myo</u> -inositol oxide or <u>myo</u> -epoxide
IM	Isomytilitol
HIM	Hydroxy isomytilitol
EP	1-Chloro-2,3-epoxy propane
EPEP	2,3-Epoxy-3-phenyl ethyl propionate
BO	1,2-Epoxy butane
PO	1,2-Epoxy propane
GG	2,3-Epoxy-1-propanol
ECT	1,2-Epoxy-cyclopentone triol 3,4,5/0
DEN	7,8-Epoxy-6-hydroxy-1,4-dioxa spiro (4,4) nonane
DSNT	1,4-Dioxa-spiro (4,4) nonane triol 6,7,8/0
OSE	Osmotic shock extract
ID-50	Inhibitory dose for 50 % growth
DNP	2,4-Dinitrophenol
NADH	Nicotinamide adenine dinucleotide
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Various nucleoside phosphates have their standard abbreviations.	

NOTES

1. Reference numbers are indicated in parentheses.
2. Formula numbers are indicated in parentheses and are underlined.
3. Cell mass is always expressed as dry cell weight.
4. The following terms are employed in the restricted sense only : auxotrophic organism, which needs exogenous myo-inositol ; prototrophic organism, which does not need exogenous MI.

R E S U M E

Le mécanisme de l'action cytotoxique d'un dérivé spiro-époxydique du myo-inositol (myo-époxyde) a été étudié sur *Schizosaccharomyces pombe*, d'autres levures et sur des bactériophages.

1. Mécanisme de la résistance chez les levures.

Les quatre levures suivantes ont des exigences différentes vis à vis du myo-inositol exogène et des sensibilités variées quant à l'action du myo-époxyde.

<u>S. pombe</u> CBS	auxotrophe pour le <u>myo</u> -inositol sensible à l'époxyde
<u>S. pombe</u> 132	auxotrophe pour le <u>myo</u> -inositol résistant à l'époxyde
<u>S. carlsbergensis</u> 9080	prototrophe pour le <u>myo</u> -inositol résistant à l'époxyde
<u>S. cerevisiae</u> 2357	prototrophe pour le <u>myo</u> -inositol résistant à l'époxyde

En présence de l'époxyde pendant les 44 heures de la culture, la souche susceptible incorpore 6 à 8 fois plus d'inhibiteur que les souches résistantes. Cependant, lorsque des homogénats de ces souches sont incubés en présence de l'époxyde radioactif, il n'y a pas de différence quant à la fixation de la substance, tant du point de vue qualitatif que quantitatif. La distribution de la radioactivité dans les diverses fractions était semblable pour les diverses souches. On a pu en conclure que la résistance à l'action du myo-époxyde est due aux différences dans la pénétration de la substance.

L'incorporation du myo-époxyde, même chez la souche susceptible, était extrêmement faible, 1,4 à 2,9 % de ce qui

était initialement dans le milieu. En augmentant la concentration de l'inhibiteur dans le milieu, on a observé une incorporation accrue dans les cellules de S. pombe CBS accompagnée d'une inhibition de croissance et d'une élongation spectaculaire. L'inhibiteur pénètre dans les cellules sans modification du noyau époxydique puisqu'on retrouvé la substance intacte dans les extraits alcooliques (80 %) de cellules inhibées et soigneusement lavées.

2. Pénétration du myo-époxyde dans les cellules de S. pombe sensibles; mécanisme de protection par le myo-inositol.

Le processus de pénétration de l'époxyde est énergie-dépendant puisque, en l'absence de glucose, elle est réduite de 27 %; de même, en présence de 2,4-dinitrophénol, on observe une inhibition de 45 %.

On a recherché si la pénétration de l'époxyde était inhibée par le myo-inositol et l'isomytilitol qui, eux, sont transportés par un système actif. Excepté durant les 20 premières minutes, où on n'a pas observé d'inhibition, une réduction de la teneur en époxyde des cellules comprise entre 6 et 15 % a été provoquée par la présence d'isomytilitol. En présence de quantités supplémentaires de myo-inositol, on a observé pendant la croissance une réduction d'au moins huit fois de l'incorporation du myo-époxyde. Au contraire, sur des homogénats, aucune réduction de la fixation de l'époxyde par le myo-inositol n'a pu être démontrée. Il est donc évident que la protection exercée par le myo-inositol n'est effective qu'au niveau de la pénétration de l'inhibiteur dans la cellule. Une fois que la substance se trouve à l'intérieur, aucune protection ne peut être obtenue par des quantités, même massives, de myo-inositol.

3. Réactivité du myo-époxyde

Le myo-époxyde réagit avec de nombreuses substances: ADN dénaturé ou non, ARN et ses composants tels que bases, nucléosides ou nucléosides phosphates. L'hydrolyse acide d'ADN

ou d'ARN traités par l'époxyde ne donne qu'un produit de réaction décelable par les méthodes chromatographiques. Sur la base de ses propriétés chromatographiques et de ses caractéristiques spectrales, cette substance est considérée comme étant un dérivé alkylé de la guanine. On peut ajouter que l'ADN traité voit son rapport guanine à adénine passer de 0,8 à 0,7 , ce qui montre bien que la réaction se fait sur le reste de guanine.

Il a été possible d'étudier quelques uns des produits de réaction du myo-époxyde avec le GMP et l'AMP. La discussion s'est portée sur les spectres ultra-violets, de fluorescence et de résonnance magnétique nucléaire.

On a constaté que l'époxyde pouvait aussi réagir en particulier avec l'imidazole, l'histidine, la nicotinamide, le NADH et encore bien d'autres substances. Le mécanisme de réaction électrophile du myo-époxyde avec les centres nucléophiles a été discutée.

4. Inhibition de croissance chez S. pombe CBS produite par divers époxydes

On a comparé les propriétés inhibitrices de 7 mono-époxydes chez S.pombe CBS. Tous, excepté le 1,2-époxy-cyclopentanone-triol-3,4,5/0, étaient capables d'exercer une inhibition de croissance accompagnée d'une élongation cellulaire, semblables à celles induites par le myo-époxyde.

5. Mécanismes moléculaires de l'action du myo-époxyde

Rapporté à l'unité cellulaire, chez S. pombe CBS inhibé à 50 % par le myo-époxyde, la quantité d'ADN est la même que chez les cellules normales, alors que les protéines et l'ARN augmentent trois fois. Les cellules inhibées ont une longueur égale à trois fois celle des cellules normales; il est alors évident que le myo-époxyde inhibe la répllication de l'ADN alors que la synthèse des protéines et celle de l'ARN ne semble pas affectée, la respiration cellulaire non plus.

Le 70 % du (^{14}C)myo-époxyde incorporé chez S. pombe CBS se retrouve dans la fraction extraite par l'éthanol 80 %. Le 8 % seulement de la radioactivité insoluble dans l'éthanol peut être extrait par de l'eau. Dans l'extrait alcoolique la majeure partie de la radioactivité se retrouve sous forme d'époxyde libre et de son produit d'hydrolyse. Une des autres substances marquées a été identifiée provisoirement par ses propriétés chromatographiques dans deux systèmes comme étant un dérivé alkylé de la guanine. Cette substance correspond aussi au seul produit de réaction obtenu à partir d'ADN ou d'ARN et de myo-époxyde. On pense que ce produit d'alkylation de la guanine provient de la dépurination de l'ADN cellulaire, ce qui provoquerait une rupture de la séquence des bases et empêcherait la réplication de l'ADN. Ce mécanisme expliquerait l'inhibition de la division nucléaire, l'élongation cellulaire et les autres effets cytotoxiques induits par le myo-époxyde chez S. pombe CBS.

Le myo-époxyde inactive le phage "fd" à ADN monocaténaire ainsi que l'action transformante de l'ADN bicaténaire du phage λ .

Au vu de ces résultats, on conclut que l'ADN est la cible déterminante pour l'action du myo-époxyde, très probablement par réaction au niveau des restes guanine.

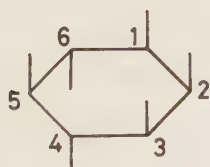
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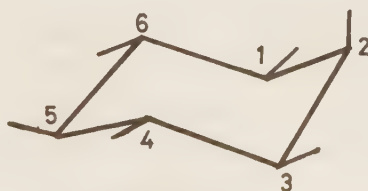
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I N T R O D U C T I O N

Since 1928, when E.V. Eastcott (1) identified one part of Bios (2) with myo-inositol (1) which she isolated from tea-leaves, not much progress has been made in the understanding of the physiological role (3-17) of this cyclitol. One of the approaches is to study its action as a growth factor in the organisms lacking the ability to synthesize it. Posternak, Schopfer and collaborators have been interested in the study of various chemical and biological aspects of this cyclitol (15-25).



Structural



Chair - form

(1) Myo-inositol

The largest portion of cellular myo-inositol is known to be incorporated in the phospholipid fractions, amongst which the mono-, di-, and tri-phosphoinositides are the most common ones(5, 11, 15, 19, 21, 24, 26). In plants and other organisms myo-inositol containing sphingolipids have been also reported (27,28).

The phospholipids are thought to be intimately associated with membrane structure and functions (6,7,11,29). A considerable amount of research has centred around the involvement of myo-inositol in cell-walls and cell-membranes (5,6,11,16). An excellent monograph on the regulatory functions of biological membranes is available (29). Matile (9) showed the incorporation of (^{14}C) inositol into the cytoplasmic membranes of inositol requiring mutants of Neurospora crassa. In presence of suboptimal amounts of myo-inositol in medium, Schizosaccharomyces pombe (23), and other inositol requiring yeasts (3,6) grow abnormally and are smaller in size.

The observation of clumps of unseparated cells in cultures grown in myo-inositol deficient medium suggests the possibility of its association with cell division processes (3,4,6). A series of papers have been published on the metabolic functions of myo-inositol, implicating nucleic acids (4,8,14). It has been reported that myo-inositol stimulates cell division in cultured carrot tissue (30). It also prevents ultra-violet and x-ray induced mutations in cells (14). These results, however, need further confirmation from other systems before any generalization is attempted regarding the role of myo-inositol in cell division and nucleic acid functions.

Posternak and collaborators synthesized a number of inositol derivatives (some important ones are shown in Table 1) and systematically tested them on myo-inositol auxotrophic organisms, Neurospora crassa "inositolless" and Schizosaccharomyces pombe (15-23,26,31-34). Out of more than sixty compounds tested, only seventeen are reported to be growth inhibitors of N. crassa "inositolless" (26,31,33) whereas only two show any significant inhibition in S. pombe (23,32,33). None of these inositol derivatives, however, show any growth promoting or vitamin-like activity.

Table 1

Biologically active myo-inositol derivatives (20,23,24,26, 31,32).

Formula number	Derivative- Formula	Inhibition Index* for	
		<u>N. crassa</u> "inositolless"	<u>S. pombe</u>
(2)	 CH ₃	30.8	22.2
(3)	 CH ₂ Br	34.4	-
(4)	 CHBr CH ₂ CH ₃	15.0	-
(5)	 CH ₂	15.6	65.6
(6)	 CH ₂	-	937.-
(7)	 CH ₂ CH ₃	16.0	No inhibition
(8)	 CH ₂ CH ₂ CH ₃	3.9	"
(9)	 CH ₂ OH	53.8	"
(10)	 CH ₂ ·NH·CH ₂ ·CH ₃	107. -	"

* Inhibition Index = molar conc. of inhibitor/ molar
conc. of myo-inositol; for 50% inhibition
(Note that lower the index, the more powerful is the inhibitor)

On the contrary, in all cases, the organisms can be protected against the inhibitory action of these derivatives, sometimes in an apparent competitive manner, if excess of myo-inositol is added in the medium before or along with the inhibitor (23). It has been pointed out that all of these biologically active derivatives of myo-inositol possess a modification at the C-2 position (33).

Myo-inositol ring (1) can exist in various conformations. The carbon skeleton of myo-inositol usually exists in the so called chair form (1). From thermodynamic considerations it is known that the most favoured conformer is the one with a minimum number of axial hydroxyl groups. In case of myo-inositol, one of the conformers will have five axial and one equatorial hydroxyls. The other conformer, thermodynamically favoured, will have five equatorial and one axial hydroxyls; the axial hydroxyl being at the C-2 position. Structurally, all of the myo-inositol derivatives will be referred to this thermodynamically stable conformation only. At the C-2 position, the substituent has an equatorial orientation. At any other position of the ring, its orientation becomes axial. A very mild inhibitory property of epi-epoxide (6) in contrast with that of myo-epoxide (5) has been explained from this point of view (33). The inhibition might have been, as well, due to a slight contamination of epi-epoxide with myo-epoxide.

As already mentioned above, Schizosaccharomyces pombe reacts more selectively towards these inhibitors than does Neurospora crassa "inositolless". S. pombe is inhibited by only two of them, namely, 2-C-methyl-myo-inositol (isomytilitol; 2) and 2-C-methylene-myo-inositol oxide (myo-epoxide; 5). Besides the growth inhibition, as measured by cell density in Klett-photometer, the surviving S. pombe cells show very remarkable modifications in shapes and sizes (Fig 1), under the influence of these two



Fig 1. Morphological modifications induced in Schizosaccharomyces pombe by inhibitors.
A : Normal cells ; B : isomylitol inhibited cells ; C : myo-epoxide inhibited cells.
Magnification, $\times 1500$.

inhibitors (20,23,32). Because of these interesting morphological modifications induced by the inhibitors, S. pombe has become a relatively more promising organism than N. crassa for further investigations of morphological and biochemical aspects of myo-inositol functions in relation with its antagonist-derivatives (15-17,22,34-36).

The morphological modifications in the surviving cells of a 50% inhibited S. pombe culture under the influence of myo-epoxide (5) and those under the influence of isomytilitol (2) can be summarized as follows (see Fig 1):

The isomytilitol cells are deformed and irregularly swollen. One or more transverse septa are present resulting in the formation of two or more cellular compartments; each compartment possesses a single nucleus. In view of the fact that normally S. pombe cells divide by fission (66), in which process the nuclear division and the formation of transverse septum are followed by separation of two daughter cells, it is evident that isomytilitol does not block the nuclear division or the separation of daughter cells (23,32). Some abnormal and additional polysaccharides are also reported to be present in the walls of the inhibited cells.

In isomytilitol treated cells, the blockage of the separation of daughter cells most likely involved membranes. Phosphoinositides are an important group of membrane-lipids. Biochemical studies soon revealed (15,19,21,22) that in isomytilitol cells a parallel incorporation of isomytilitol (parallel to the myo-inositol incorporation) takes place in the phospholipids of the cells. Thus, besides the normal inositol-phospholipids, a large amount of abnormal isomytilitol-phospholipids also accumulates in the cells. These abnormal isomytilitol-phospholipids may be responsible for the abnormal morphology, whereby, though the cellular division proceeds almost normally the daughter cells do not

separate.

On the other hand, the myo-epoxide (5) inhibited cells show a markedly different morphology. No transverse septa are formed (or these are incompletely formed), and the cell continues to grow and elongate; sometimes as much as 10-20 -fold its normal length (23). No nuclear division takes place. These giant cells possess only one nucleus each (20,23,35). Thus, it is obvious that both the nuclear division and the septum formation are inhibited due to the influence of myo-epoxide. As with the isomytilitol inhibition, an excess of myo-inositol added to the medium at the beginning of growth is shown to over come the myo-epoxide effects (23). In contrast with the isomytilitol behaviour, however, little or no incorporation of myo-epoxide in the phospholipid fractions of the inhibited cells is found (15,19,22). Furthermore, myo-epoxide inhibited cells accumulate more myo-inositol in the form of phosphoinositides than does the normal cells (15,21). Hence, the possibility of an abnormal-cyclitol-phospholipid mediated membrane intervention in the production of morphological deformities in myo-epoxide cells becomes much less likely than in the case of isomytilitol cells.

An interesting phenomenon of antagonism between the two inhibitors, myo-epoxide and isomytilitol, has been also reported (23). In presence of normal amount of myo-inositol, if a large excess of myo-epoxide and small, uninhibitory amount of isomytilitol are simultaneously added to the medium at the beginning of growth, the expected amount of growth inhibition due to myo-epoxide in S. pombe is decreased. In other words, isomytilitol can protect cells against the myo-epoxide action, though, upto a certain extent only. If larger amount of isomytilitol is added to the medium, growth inhibition occurs, as well, from isomytilitol itself.

Excellent reviews on the biochemistry and the regulatory mechanisms of cell division have appeared (37,38). Ionizing radiations and the radiomimetic compounds (39) inhibit cell division at concentrations less than those required to inhibit growth (40-43). Observing the effects of a great many chemicals, including mono-, and di-functional alkylating agents, upon cell division and growth in E. coli B and S. cerevisiae, it has been concluded that whenever division inhibition is encountered, cell elongation is also consequently evoked (40). Brown and Hough reported the production of elongated cells of S. cerevisiae under conditions which limit nitrogen supply in the medium (44).

Biological alkylating agents are known to react with cellular DNA (45-58). Some of these caryotoxic agents, especially, the bi-functional ones, form cross-links in the opposite strands of DNA (39,59). This results in an inhibition of the rate of DNA synthesis. The failure of the cell to replicate its DNA leads to mitotic delay and cell enlargement in HeLa cells treated with mustard gas (51). In E. coli K 12, septum formation is prevented following the arrest of DNA synthesis and this results in the formation of elongated cells (60). A block in DNA replication may be produced either in the initiation steps or the subsequent propagation (43). Recently, it has been pointed out that the cells in late G-1 and S phases of the mitotic cycle are particularly sensitive to alkylating agents (43, 51,61).

Schizosaccharomyces pombe (62-65) is a yeast depending for its growth on external supply of myo-inositol. Like bacteria, it divides by fission (66). Studies on the cell-cycle of this yeast has been published (67,68). Besides the myo-inositol derivatives discussed earlier (Table 1), a number of other chemicals, including some

nucleic acid base analogs, antibiotics and alkylating agents, have been also tested for growth inhibition and induction of morphological modifications in S. pombe (34). Two out of them, namely, 8-azaguanine and β -propiolactone, induce the production of uninucleate elongated cells without any transverse septa; similar to those produced by myo-epoxide (see Fig 1). 5-fluoro-uracil and actinomycin also produce elongated cells, though, some of them are compartmented by the presence of 2 to 3 transverse septa (34). More recently, cell elongation induced by restrictive temperature in the temperature sensitive mutants of S. pombe has been also reported (69).

The foregoing considerations led us to form the working hypothesis that the cellular site of myo-epoxide action is on nucleic acid molecules, more specifically on the DNA.

Epoxides, in general, are well known biologically active compounds (39,46,70-76). Many of them have been reported as cytotoxic alkylating agents, mutagens, carcinogens, teratogens and even carcinostatic compounds (39,46,73). Though the precise molecular mechanism of mutagenic and cytotoxic lethal actions of biological alkylating agents is not yet fully understood, the accumulated evidence strongly suggests that the reaction of such agents with nucleic acids may lead to the inhibition of normal biochemical functions of the latter (48,75,77-89). In view of the involvement of genetic material, and as far as the cellular economy is concerned, DNA is considered to be the most critical target molecule of alkylation (39,46,54,56).

A nucleic acid molecule offers several possible sites of alkylation, for example, the purines and the pyrimidine bases (75,76,90-105), and the phosphoryl groups (45,85,89,106-109). The N-7 atom of guanine in DNA is one of the principal sites of attack by alkylating agents including

epoxides (48,76,78,79,87,94,104,110-113). Studies on the alkylation of other sites, like, N-3, N-1, and O-6 in guanine; N-1, N-3, and N-7 in adenine; and N-1 in cytosine and in uracil (thymine) are also available (46,50,56, 91,95,96,102,104,114,115). In addition to the nucleic acids and their components, other biologically important groups, like, imidazole, imino, thiol, and sulphhydryl are also vulnerable to the attack by electrophyllic agents (46,90, 104).

If nucleic acids were the target molecules in vivo for attack by alkylating agents, various bacterial phages would be simpler models for investigating their biological effects. Indeed, biological alkylating agents have been reported to inactivate the bacteriophages and the transforming principles (39,77,116-126).

The alkylated purines become labile and gets easily removed from DNA (45,48,51,57,58,78,79,81,83-85,87). This results in depurinated DNA, which in turn may become incapable of acting as a normal template (51,55,75,80,86). An inhibition of DNA replication would result in nuclear division inhibition and filament formation (43,51,60,69).

There seemed, however, one apparent ambiguity in adopting this working hypothesis, which considered nucleic acids as the cellular targets of myo-epoxide action. It appeared highly improbable to locate the protective action of myo-inositol (23) against myo-epoxide on the nucleic acids.

In myo-epoxide inhibited S. pombe cultures, almost all of the uptaken myo-inositol is found in the phospho - lipid fraction and none of it is shown to be associated, in any way, with cellular nucleic acids. Complicating the situation still further was the observation that myo-epoxide inhibited cultures contain more myo-inositol phospholipids per cellular unit than does the normal

cultures (15). In other words, the possibility of any protective action of myo-inositol-lipids against the action of myo-epoxide in S. pombe may be ruled out.

We, therefore, undertook experiments to clarify this apparent ambiguity and to study the molecular mechanism of myo-epoxide in S. pombe and other yeasts.

One of the mechanisms through which cells resist the action of drugs is known to be due to its decreased uptake from the medium (65,127-138). The evidence presented in the present report shows that the protective action of myo-inositol, in S. pombe CBS, against the action of myo-epoxide is due to the diminished uptake of the inhibitor from the medium by the cells. Once myo-epoxide has entered the cell no protection is offered by myo-inositol against the attack by the former at the reactive cellular sites. Further evidence is produced which suggests that the most likely critical cellular target for myo-epoxide attack is DNA; and that its cytotoxic and growth-inhibitory-lethal effects are probably due to the resulting depurination from the myo-epoxylated DNA.

Experiments were also undertaken to understand the chemical reactivity of myo-epoxide with nucleic acid components and other biologically important molecules. The results are discussed.

We have further shown that myo-epoxide inhibited the bacteriophages and the transforming activity of the phage-DNA. These facts support the hypothesis that the site of myo-epoxide action is on the nucleic acid molecules. No protective action of myo-inositol is observed in the inactivation of single stranded DNA phage "fd" by myo-epoxide. All these results are discussed in the light of available evidence and with a view to elucidate the molecular mechanism of the action of myo-epoxide.

M A T E R I A L A N D M E T H O D S

1. Organisms utilized:

The following organisms were employed in these experiments:

Yeasts:

i) Schizosaccharomyces pombe Lindner, liquefaciens, Osterwalder CBS , 1042 ; the Centralbureau voor Schimmelcultures, Yeast Division, Delft, Netherlands.

ii) Schizosaccharomyces pombe NTCC 132; kindly supplied by Prof. J.M. Mitchison, Univ. of Edinburgh, Scotland.

iii) Saccharomyces carlsbergensis ATCC 9080

iv) Saccharomyces cerevisiae Hensen 2357 (Bern)

Bacteriophages:

i) Phage λ (E. coli), C.O. stock 39; DNA-double-stranded phage.

ii) Phage "fd" (E.coli), M 36/11 ; DNA -single-stranded.

iii) Phage λ .434 SUS A⁻ SUS R⁻ ; helper phage for the DNA transformation experiments.

Bacteria:

i) Escherichia coli 991

The bacteriophages and the E. coli strains were kindly made available from the Institutes of Molecular Biology, University of Geneva.

2. Culture conditions for yeasts:

The stock cultures were maintained on solid malt-yeast-agar medium on slants at 4°. The cultures were replicated on fresh slants every month. Before inoculation in the Pennington medium (22,23,139), the cells were grown in an "intermediary medium" (22). To do this, the cells from stock cultures were transferred to 20-50 ml intermediary medium, incubated for 24 h at 29-30°. Cells from log phase were harvested by centrifugation and were washed twice with sterile water. The washed cells were diluted with sterile water to give an optical density of 50 klett units (Klett-Summerson photoelectric colorimeter; green filter number 54). 0.1 ml of this diluted cell suspension (about 10^6 cells) was employed for inoculation for 10 ml Pennington medium in 50 ml Erlenmeyer flasks. All operations were done under sterile conditions. Incubation was done at 29-30°. When larger amounts of medium were used, the inoculum was scaled up in proportion.

3. Chemicals:

Following special chemicals were employed during the course of these studies:

i. 2-C-Methylene-myo-inositol oxide (Myo-epoxide).

Myo-epoxide was synthesized from 2-keto myo-inositol by the action of diazomethane (140,141). The purity of myo-epoxide was checked by descending chromatography with solvent system A-14 at 4°. It gave only a single spot which was revealed by alkaline silver nitrate, followed by fixation with $\text{Na}_2\text{S}_2\text{O}_3$ (142).

ii. 2-C-(^{14}C) Methylene-myo-inositol oxide.

(^{14}C) Myo-epoxide was prepared by Dr. Falbriard by reacting (^{14}C) diazomethane with the pentaacetyl-2-keto-myo-inositol. After removal of the acetyl groups the product was purified by paper chromatography in the solvent system A-14. The specific activity of the purified product

was found to be 764 CPM per μg .

iii. Other epoxides.

- a. 1-Chloro-2,3-epoxypropane (dl) : EP
 - b. 2,3-Epoxy-3-phenyl ethyl propionate : EPEP
 - c. 1,2-Epoxy butane : BO
 - d. 1,2-Epoxy propane : PO
 - e. 2,3-Epoxy-1-propanol : GG
 - f. 1,2-Epoxy cyclopentone triol 3,4,5/0 : ECT
 - g. 1,4-Dioxa-6-hydroxy-7,8-epoxy-(4,4) spiro
nonone : DEN
 - h. 1,4-Dioxa-(4,4) spiro nonone triol 6,7,8/0: DSNT
- Chemicals (a) to (e) were purchased from Fluka, Switzerland, and those from (f) to (h) were synthesized in

this laboratory by Dr. F. Cocu.

iv. Pantoyl lactone (Formula: 19; p 61), was a kind gift from Dr. A. Loveless of the Chester Beatty Research Institute, London, U.K.

4. Sterilization:

Glass-ware, media and water were sterilized by autoclaving at 120° (1 - 1.2 kg/ cm^2 pressure) for 15-20 min.

Myo-epoxide, other epoxides and pantoyl lactone were sterilized by filtration through fritted glass filter number G-5, or through Millipore filters. The filtration assembly was sterilized beforehand by autoclaving.

5. Composition of Pennington medium (22,23,139):

Glucose	10 g	Vitamin free casein hydrolysate	1 g
Asparagine	2 g	Calcium pantothenate	1 mg
$(\text{NH}_4)_2 \text{SO}_4$	2 g	Pyridoxine	1 mg
$\text{KH}_2 \text{PO}_4$	2 g	Thiamine	1 mg
$\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g	Nicotinic acid	1 mg
CaCl_2	0.25 g	Biotine	1 μg

Distilled water was added to make 1000 ml. The medium was supplemented with 1 mg myo-inositol per litre.

6. Measurement of growth:

It was done by measuring the optical density (O.D.) in a Klett-Summerson photoelectric colorimeter using number 54 filter. Biophotometer measurements have been related to the cell suspension mass and not to the number of the cells (23). As such, the weight of the cells will depend on the extent of inhibition, cell length and the density of the cell-mass (23). The growth inhibition experiments were done in specially designed 200 ml Erlenmeyer flasks; each such flask had at its bottom a Klett-tube attached, which enabled direct readings in the photometer.

7. Morphological modifications:

Changes in cell-morphology were observed through a phase-contrast microscope.

8. Uptake experiments:

In general, a known amount of (^{14}C) myo-epoxide was added in 10 ml Pennington medium to which about 10^6 cells were inoculated. The taken up amount was measured by counting the radioactivity uptaken by the cells at the end of 44 h growth.

Uptake experiments, for shorter periods, were done in the following manner. Cells from the log phase of growth were harvested, washed with water and were suspended in the specified medium or the buffer solution containing a known amount of (^{14}C) myo-epoxide. At the end of the incubation period, cells with taken up radioactivity were harvested, washed and an aliquot suspended in the counting solution; the radioactivity was measured by usual counting procedure. An aliquot from the left-over medium was also counted.

9. "Fixed" myo-epoxide in the cells:

The cells with the taken up radioactivity were harvested, washed in water and resuspended in 50 ml of fresh

Pennington medium which contained a large excess (6 mg) of non-labeled myo-epoxide. A drop of toluene was added. The cell suspension was incubated at room temperature with gentle stirring for 2 h. Based on the earlier efflux studies (25), where 30 min have been found to be sufficient time for out-flow of all of the free internal myo-inositol, a 2 h exchange period was chosen, for present experiments, during which period all of the unbound (^{14}C) myo-epoxide would get exchanged by the non-labeled compound. After this incubation period, the cells were again harvested, washed and counted for the remaining activity. The remaining radioactivity was taken as the "fixed" myo-epoxide in the cells.

10. Osmotic Shock Extract (OSE):

Essentially, the method of Neu and Heppel (143) was used. The cells were harvested by centrifugation. No washing was performed. The cells were then suspended in 20.6% sucrose solution in 0.03 M tris-HCl buffer (pH 7.1), which contained additionally 0.15 ml of 0.2 M EDTA solution. The suspension was incubated at 30° for 15 min followed by a rapid cooling in ice. It was then centrifuged at 12 100 g for 10 min in cold. The supernatant was discarded. The cells were resuspended in the same volume of ice-cold water and kept for 10 min with intermittent shaking. They were again centrifuged. This time the supernatant was collected and designated as the Osmotic Shock Extract (OSE). The sedimented cells were referred as the Shocked Cells. The shocked cells were checked by staining with the neutral red and were found to be viable.

11. Preparation of cell homogenates:

The cell homogenates were prepared by crushing the frozen cell-suspensions in a pre-cooled (about -50°) Eaton Press (144) at 12-14 tonnes. The disintegration of cells

was controlled under a phase-contrast microscope. Generally speaking, more than 90% cells were found to be broken after the operation.

12. Fractionation of (^{14}C) myo-epoxide labeled metabolites:

The cells grown in presence of (^{14}C) myo-epoxide were harvested, washed 3 times with water and the homogenates were prepared in the Eaton Press. The fractionation scheme is given in Fig 2, and is described below. All operations were carried out in cold.

The 80% Ethanol Extract.

To the homogenate, absolute ethanol was added to make 80%. It was then centrifuged at 27 000 g for 15 min. The supernatant was designated as the Ethanol Extract.

Water Extract.

The ethanol insoluble pellet was thoroughly resuspended in water and was kept in cold room for 12 h. The suspension was centrifuged in Spinco at 59 000 g for 90 min. The supernatant was referred to as the Water Extract.

Residue.

The remaining pellet was referred to as the Residue. In one of the experiments, the residue was further hydrolysed with 1 M KOH at 30° for 12 h. Two fractions were obtained, namely, the Alkali hydrolysable residue and the Alkali insoluble residue.

13. In vitro reaction of biologically important compounds with myo-epoxide :

Nucleic acids (DNA and RNA; commercial) and nucleic acid components, various N-heterocyclic compounds, amino acids, and carboxylic acids were made to react with myo-epoxide. Unless otherwise mentioned, the reactions were carried out in water at 30-37° for 12-36 h. Usually, the reactants were added in equimolar ratios, but in some

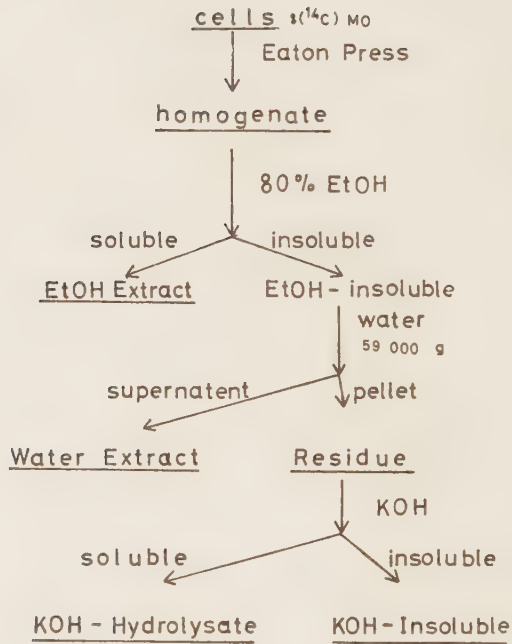


Fig 2. Fractionation scheme for isolating the myo-epoxide metabolites

cases an excess of myo-epoxide was used. The reaction products were separated by chromatography or electrophoresis. The spots were located in UV light.

Because of the poor water solubility of nucleic acid bases, they were dissolved in 0.005 N KOH and reacted with myo-epoxide. Though the method was far from being simulative of the physiological conditions, at least, it gave an idea of the comparative reactivity of these bases towards myo-epoxide (145,146).

Reaction of myo-epoxide with RNA:

Low molecular-weight yeast RNA was used. To a solution of 100 mg RNA per 14 ml of 5.8% NaCl, 51 mg of myo-epoxide was added (pH adjusted to 6.8). Reaction was allowed to proceed at room temperature for 27 h with constant stirring. Throughout the reaction period an increase in pH of the solution was observed. The pH was kept constant within a range of 6.8 to 8.2. At the end of this period, the reacted RNA was precipitated by adding ethanol to make 80%.

Reaction with calf-thymus DNA:

19 mg of the sodium salt of calf-thymus DNA (Fluka) was dissolved in 4.7 ml of 1.25 M sodium acetate buffer, pH 5.9 (ref: 104), to which 100 mg myo-epoxide was added. The mixture was incubated for 48 h at 30°. The reaction was terminated by precipitation of the reacted DNA with ethanol to make 80%.

Acid hydrolysis.

Myo-epoxide-reacted DNA and RNA, each separately was further hydrolysed in 1 N HCl at 100° for 1 h. The hydrolysis products were separated chromatographically.

14. The methods of separation:

The following chromatographic and electrophoretic

methods of separation were employed:

- A. Paper chromatography (ascending/descending) Whatman 1 or 3 MM paper.
- C. TLC silica gel HF -254
- D. TLC cellulose
- F. Sephadex G-25 Columns
- G. Electrophoresis; Whatman 1 paper; 1000 volts, 1h.

Developing solvents employed.

All proportions mentioned are v/v, except when otherwise mentioned.

- 1. 1 M ammonium acetate-ethanol (Merck), 3:6
- 2. 1 M ammonium chloride-ethanol (Merck), 3:6
- 3. Isopropanol-ammonia-water , 140:5:60
- 3(a). Isopropanol-ammonia-water , 100:5:10
- 4. Methanol-HCl-Water , 7:2:1 (or 6.5:2.5:1)
- 5. n-Butanol-acetic acid-water , 3.5:0.5:1.5
- 6. Chloroform-methanol, 2:1
- 7. Chloroform-ammonia-methanol-water , 55:8:65:21
- 8. Iso-butyric acid-ammonia-water , 57:4:39
- 9. Phosphate buffer (0.1 M; pH 6.8)-ammonium sulphate-n-propanol , 100:60:2 (v/w/v)
- 10. Water
- 11. 0.85% NaCl solution (w/v)
- 12. 0.03 M / 0.08 M citrate buffer , pH 3.5
- 13. n-Butanol-water , 4:1 (saturated butanol layer from the top was used)
- 14. Acetone-water , 4:1 (0° to 4°)

15. Measurement of radioactivity:

Radioactivity was measured in Beckman liquid scintillation counter, model LS 250, by counting the samples in the following solution: naphthelene - PPO - p-dioxane, 100 g - 5 g - 1000 ml. Whenever necessary to keep the aliquot homogeneously in suspension, the above counting solution also contained 4 g Cab-O-Sil per litre.

Location of radioactive spots on paper chromatograms was done by scanning the strips through Actigraph III (Nuclear-Chicago).

16. Chemical analyses:

Protein:

Protein was estimated using the method of Lowry et al (147), with purified casein as standard.

Sucrase activity in the OSE (148) :

2.5% sucrose solution was prepared in 0.01 M sodium acetate buffer, pH 4.6 . To 4 ml of this solution, 2 ml of the osmotic shock extract (OSE) was added and incubation was done at 20° for 2 h. Hydrolysis was terminated by heating the tubes in a boiling water bath for 10 min. The glucose in the hydrolysate was estimated following the standard "Glucostat" (Worthington) procedure.

Nucleic acids:

Extraction from lyophilized cells was done by perchloric acid according to the method of Ogur and Rosen (149).

DNA estimation:

DNA was estimated in the extracts by the diphenylamine method (150), using calf-thymus DNA or deoxyadenosine as standard. Whenever deoxyadenosine was used as standard, the readings were multiplied by 2.5 to obtain corresponding DNA values. The intensity of the colour was measured at 590 nm.

RNA estimation:

RNA was estimated by measuring the absorbance of an appropriate dilution of the extract at 260 nm in comparison with commercial RNA (Fluka) as standard.

Phosphates :

The amount of phosphate in a sample was estimated after mineralization with sulphuric acid, according to the procedure of Fiske and Subbarow (151). For detection of phosphates on paper or thin layer chromatograms, a molybdate-perchloric spray was used (see ref: 152).

17. Respiration experiments:

Respiration experiments were performed in Warburg apparatus according to the classical method (153). Homogenate of normal S. pombe CBS cells from log phase was prepared in 0.1 M phosphate buffer (pH 5.8). The main body of the flask contained an aliquot of the homogenate (117-227 mg; dry weight), 1.8-2.7 mg glucose and either myo-inositol (18 mg), myo-epoxide (4 mg) or both. In addition to this, each flask also contained a co-enzyme solution whose composition has been described in the ref: 140. The oxygen consumption and the carbon dioxide evolution were measured for a period of 90 min.

18. Inactivation of bacteriophages and transforming principle by myo-epoxide:

Inactivation of phage λ :

The stock culture of phage λ C.O. Stock 39 was diluted in TM buffer (Tris $10^{-2}M$ + $MgSO_4$ $10^{-3}M$; pH 7.0) to give a concentration of 6×10^9 phage particles per ml. They were then incubated at 30° for 30 min with varying concentrations of myo-epoxide. Appropriate dilutions of these treated phage particles were plated with E. coli 921 on soft agar plates. Plaque forming units (PFU) were recorded after incubating the plates over-night at 37°.

Inactivation of DNA "A" from phage λ :

Appropriate dilutions (10^9 DNA particles /ml) of the DNA particles in either TCM buffer (Tris-calcium-magnesium 10^{-2} M buffer, pH 8) or Tris 10^{-2} M + EDTA 10^{-3} M buffer, pH 7, were incubated with varying concentrations of myo-epoxide at 30° - 37° for 30-60 min. The treated DNA suspensions were further incubated at 37° for 30 min with the "bacterial competents" - E. coli 921 prepared specially with the helper phage λ . 434 SUS A⁻ SUS R⁻ for transformation. Appropriate dilutions from these incubation mixtures were plated and the PFU were counted after further overnight incubation at 37° .

Inactivation of single stranded DNA phage "fd":

Appropriate dilutions of phage particles were incubated with myo-epoxide in TM buffer, pH 7.0. Incubation was done at 37° for 30 min. The treated phages were plated on suitable medium with E. coli 991. After overnight incubation, at 37° , the PFU were counted.

R E S U L T S

1. Growth inhibition in yeasts by myo-epoxide.

Earlier studies (20,23,32) have revealed that myo-epoxide inhibited the growth of Schizosaccharomyces pombe CBS and induced morphological modifications in the surviving cells. It was, therefore, interesting to check whether still another strain, S. pombe NTCC 132, was equally susceptible to this epoxide.

To begin with, the myo-inositol requirement of each of these strains was checked and found to be analogous (Fig 3). Still another yeast, Saccharomyces carlsbergensis 9080, showed no exogenous requirement of myo-inositol for its growth of 44 h. In absence of myo-inositol, its development was, however, retarded during early phase of its growth (results not shown). This showed that this yeast is prototrophic for myo-inositol.

These three strains were taken as models to compare the inhibitory effects of myo-epoxide. Table 2 summarizes the results. It was observed that, whereas 0.37 mM of myo-epoxide produced 54% growth inhibition in the susceptible strain (CBS) of S. pombe, as much as 20 times this dose produced only 13% growth inhibition in the resitant (NTCC 132) strain. S. carlsbergensis, a prototrophic organism for myo-inositol, was similarly found to be resistant towards myo-epoxide action. About 14 times the ID-50 (Inhibitory Dose to produce 50% growth inhibition) for the susceptible strain CBS, produced only 18% growth inhibition

in it. Morphological changes induced by myo-epoxide in these yeasts are also interesting to be considered. It induced uninucleate, elongated cells in the S. pombe strains, while S. carlsbergensis cells remained either normal or were found to be slightly swollen in appearance.

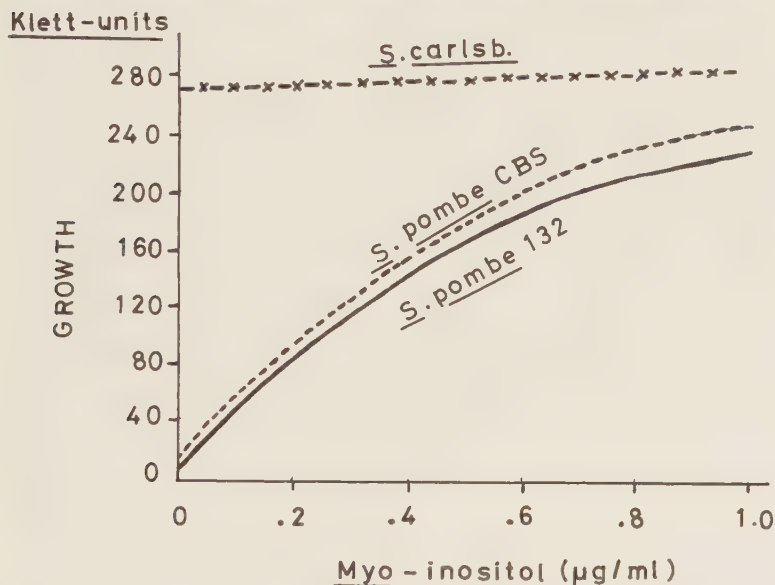


Fig 3. Growth (44h) of yeasts in presence of myo-inositol in Pennington medium.

Table 2

Growth inhibition and morphological modifications in yeasts induced by myo-epoxide; Pennington medium having 1 µg myo-inositol per ml.

Yeasts	<u>myo</u> -epoxide mM	Growth %	Cell morphology	Cell avg.length, µ
<u>S. pombe</u> CBS	0.37	46	Elongated	25.2 (8.7)*
<u>S. pombe</u> 132	0.30	100	Normal	11.8 (10.4)
<u>S. pombe</u> 132	7.29	87	Sl. Elong.	16.6 (10.4)
<u>S. carls.</u> 9080	5.21	82	sl.swoll- -en	- -

* Figures in parentheses indicate the normal avg. lengths of the cells.

2. Uptake of (^{14}C) myo-epoxide by different yeast strains during growth.

One of the ways by which microbial cells resist the action of drugs has been shown to be due to the decreased uptake of the drug from the medium (134-136). It was, therefore, investigated, whether the resistance to myo-epoxide action in yeasts could be correlated with differences in its uptake by the cells. Table 3 shows the validity of this hypothesis. The susceptible strain, CBS, took up about 6-fold more myo-epoxide, at the ID-28, than did the resistant yeasts. The latter did not show any growth inhibition either, under these conditions. Still another yeast, Saccharomyces cerevisiae (prototrophic for myo-inositol), which also resisted the myo-epoxide action, similarly showed a very low uptake of the inhibitor.

Increasing amounts of myo-epoxide in the medium has been shown to produce increasing growth inhibition in the susceptible yeast S. pombe CBS (23). We considered whether increasing concentrations of myo-epoxide in the medium, likewise, resulted in an analogous increase in its uptake by the cells. The results summarized in Table 4 are self-explanatory. The more the epoxide was offered in the medium, the more of it was taken up and got fixed in the cells. It was, nevertheless, interesting to observe that the percentage of the offered amount of radioactivity which was taken up by the cells remained almost constant (2.4 to 2.9 %) in all treatments, except when a very low amount of myo-epoxide (corresponding to, 211×10^3 CPM) was offered in the medium. In this latter case, only 1.4% of the offered amount was uptaken.

These results (Table 4) also indicate that only a very small percent (1.4 to 2.9) of the total offered

Table 3

Uptake of (^{14}C) myo-epoxide by yeasts during growth (44 h)
 Pennington medium, in presence of 1 μg myo-inositol per ml.
 Values are reported for 10 ml cultures. (^{14}C) myo-epoxide
 offered : 421 000 CPM.

Yeasts		Uptake per mg	Growth	
		cells.(CPM)	mg cells/10 ml	in %
<u>S. pombe</u>	CBS	1 770	6.4	72
<u>S. pombe</u>	132	311	9.0	100
<u>S. carlsb.</u>	9080	307	9.6	100
<u>S. cerevis.</u>	2357	261	9.9	100

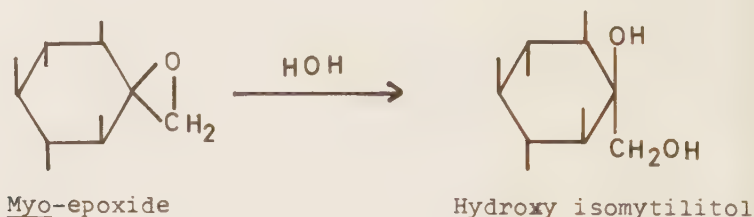
Table 4

Uptake of (^{14}C) myo-epoxide by S. pombe CBS during growth.

Pennington medium, in presence of 1 μg myo-inositol per ml.
 Values are reported for 10 ml cultures.

Offered in medium	Uptaken by cells		Total uptaken amount expressed as % of the initial counts offered	Fixed radio- activity in cells; as % of uptaken	Growth of the culture %
CPM. 10^{-3}	CPM				
	per mg cells	per unit cell			
	CPM (CPM $\times 10^6$)				
211	400	25	1.4	87	85
422	1 570	131	2.6	80	72
632	3 190	290	2.9	83	65
843	4 220	469	2.4	80	54

myo-epoxide was taken up by the cells. A chromatographic analysis of the left-over medium revealed two peaks, one that of the myo-epoxide (59 %) and the other one corresponding to its hydrolysis product, that is, hydroxy isomytilitol (41 %).



From 80 to 87% of the uptaken radioactivity was found to be "fixed" in the cells (Table 4). The remaining, 13 to 20 % of it could be effluxed out when these loaded cells were shaken with non-labeled myo-epoxide.

3. Protection by myo-inositol against the action of myo-epoxide.

Myo-inositol has been shown to protect S. pombe CBS cells against the action of myo-epoxide, if an excess of the former is added in the medium before or along with the inhibitor (23). A logical corollary to our present findings, that the resistant strain 132 took up significantly lesser amount of myo-epoxide than did the susceptible strain CBS (Table 3), was to inquire if myo-inositol similarly protected the cells by reducing the uptake of myo-epoxide or did it do so by competing with the inhibitor for some site of action within the cell.

Inhibition of uptake of myo-epoxide by myo-inositol.

At ID-50 of myo-epoxide, when 10 times more than the optimal dose of myo-inositol (that is, 10 μ g/ml instead of 1 μ g/ml) was added in the medium, a complete disinhibition of the culture was achieved (23). The results reported in Table 5 show that under these conditions myo-inositol decreased the uptake of myo-epoxide by about 9-fold in the case of susceptible strain. Simultaneously, it also protected the cells against the growth inhibition and other effects. For the sake of comparison, the data for the uptake by S. cerevisiae are also re-reported in Table 5.

Table 5

Protection by myo-inositol against the action of myo-epoxide in S. pombe CBS by decreasing the uptake of (14 C) myo-epoxide during growth (44 h). Values for 10 ml cultures are reported.

Yeasts	Added in the medium.		(14 C) <u>myo</u> -epoxide uptaken / mg cells	Growth
	<u>myo</u> -inositol	<u>myo</u> -epoxide		
	μ g	CPM $\times 10^{-3}$	CPM	%
<u>S. pombe</u> CBS	10	421	1 770	72
<u>S. pombe</u> CBS	100	421	189	100
<u>S. cerevis.</u>	10	421	261	100

In another experiment, the uptake of (14 C) myo-epoxide by the CBS strain during growth was measured over a range of increasing concentrations of myo-inositol in the

medium and the data are correlated with growth (Fig 4). It is evident from the results that when myo-inositol concentration was increased in the medium, the amount of the uptaken myo-epoxide decreased, and a corresponding growth disinhibition was obtained. It was noted with interest that although, the suboptimal level of myo-inositol ($0.5 \mu\text{g/ml}$) in the medium produced a growth inhibition, it did not, however, favoured myo-epoxide uptake, which in this case remained only 45 % of the maximum achieved in the experiment.

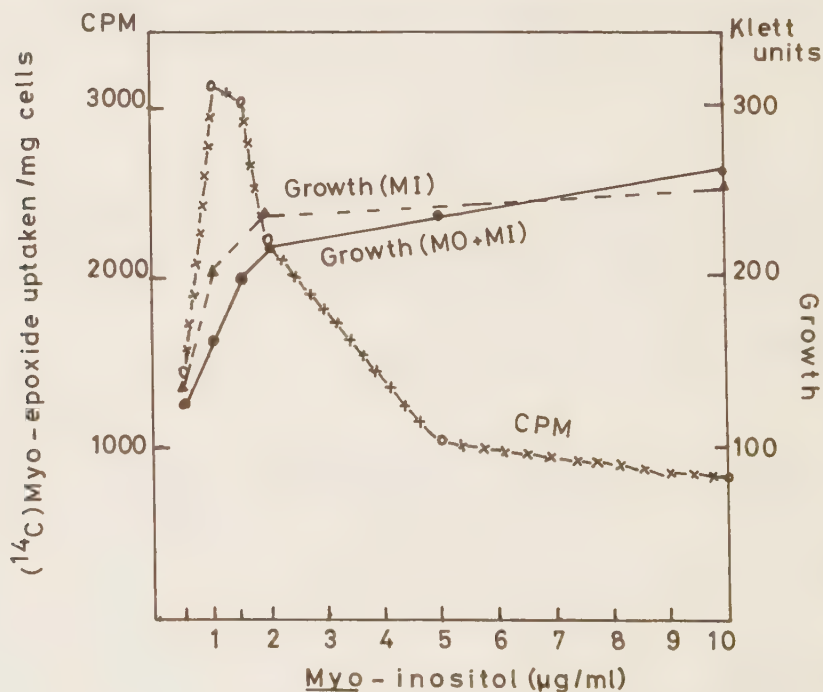


Fig. 4 . Protection by myo-inositol against the action of myo-epoxide in S. pombe CBS during growth (44 h) by decreasing the uptake of the latter. The cells were grown in presence of 640×10^2 CPM of (^{14}C) myo-epoxide per ml medium plus increasing amount of myo-inositol as shown in the Fig.

4. Uptake of myo-epoxide by cell free systems.

Further evidence was collected which show that the resistance to the action of myo-epoxide in yeasts is due to the permeability differences, and that the protection offered by myo-inositol to the susceptible strain is also due to a diminished uptake of the former from the medium.

Cell homogenates of the susceptible and the resistant strains were prepared by Eaton Press. The homogenates were incubated with (^{14}C) myo-epoxide in presence or in absence of myo-inositol at 30° for 12 h. A drop of toluene was added. Table 6 summarizes the results, which show no significant difference in the amount of the radioactivity bound by the homogenates of the resistant and the susceptible strains. Similarly, there was no decrease in the uptaken amount of myo-epoxide due to the presence of additional myo-inositol in the incubation mixture. The 80 % ethanol extracts from the incubation mixtures showed exactly super-imposable chromatographic patterns which thereby imply that same reaction products were formed in all cases (Fig 5).

The preparation of homogenate may disrupt the internal cellular organization. This fact should be taken in consideration while interpreting these results.

Table 7 shows the distribution of radioactivity from (^{14}C) myo-epoxide in various fractions obtained after its in vitro reaction with homogenates from the resistant and the susceptible yeasts. About 99% of the uptaken activity was found in the ethanol extract fraction. The water extract and the residue, each contained about 50% of the remainder radioactivity.

The presence of a permeability barrier in resistant strains is strikingly clear. Homogenates in all cases took up from 35 to 44 % of the offered myo-epoxide (Table 6), in contrast with a maximum uptake of 2.9% attainable in the

in vivo experiments, even by the susceptible yeast S. pombe CBS (Tables 4 and 5).

Table 6

Uptake of (^{14}C) myo-epoxide by yeast homogenates. The values are reported per ml incubation mixture.

Homogenate from the yeasts	Myo-inositol added in the incubation mixture	(^{14}C) <u>myo</u> -epoxide		
		offered per mg homoge- nate	uptaken per mg homoge- nate	Uptaken as % of the offered
	ug	CPM	CPM	%
<u>S. pombe</u> CBS	0	6 730	2 760	41
<u>S. pombe</u> CBS	14	6 730	2 940	44
<u>S. pombe</u> 132	0	6 730	2 355	35
<u>S. carlsb.</u> 9080	0	6 730	2 355	35

Table 7

Distribution of radioactivity in various fractions obtained from the homogenates after reaction with (^{14}C) myo-epoxide.

Homogenate	Myo-inositol* ($\mu\text{g}/\text{ml}$)	Percent radioactivity in in the fractions.			
		Ethanol	Ext. Water	Ext. Residue	
<u>S. pombe</u> CBS	0	99.1	0.5	0.4	
<u>S. pombe</u> CBS	14	98.6	0.6	0.8	
<u>S. pombe</u> 132	0	99.2	0.4	0.4	
<u>S. carlsb.</u>	0	98.0	1.0	1.0	

* Myo-inositol added in the incubation mix. see Table 6.

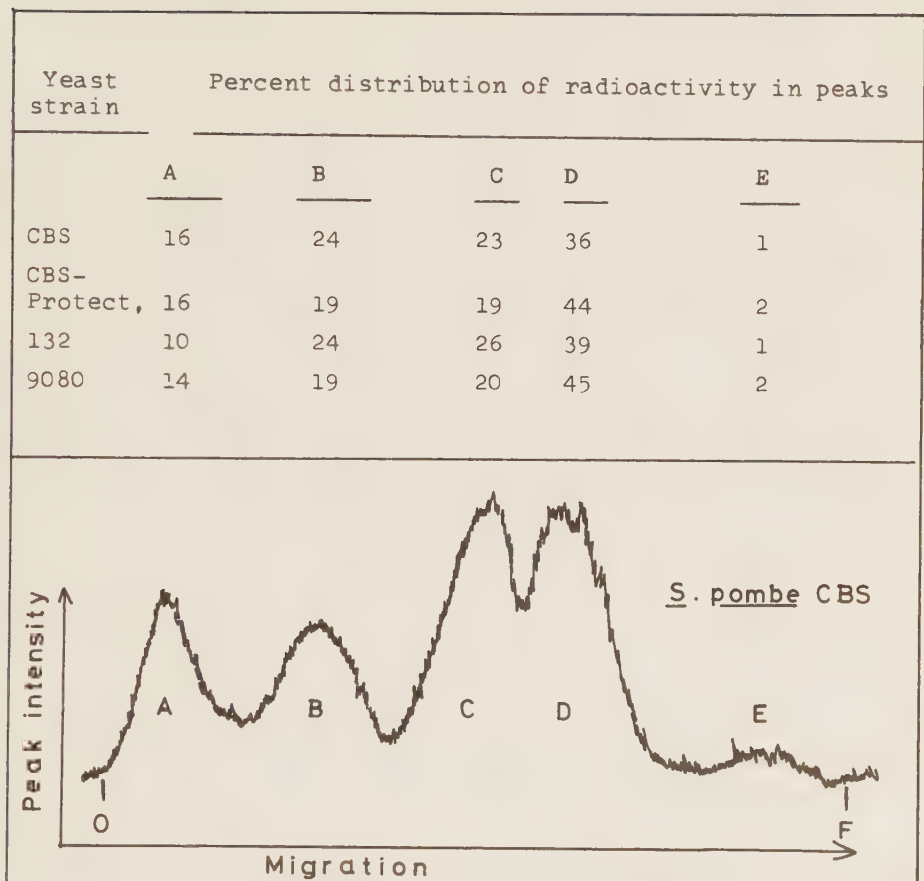


Fig 5. Radioactivity tracing of paper chromatogram of the 80 % ethanol extract from cell homogenate - myo-epoxide reaction products (ascending chromatography; system A-1). Chromatogram tracings from various homogenate - myo-epoxide reactions were exactly superimposable, hence only one is reproduced here. The percent distribution of radioactivity in different peaks for all yeasts are reported.

5. Further observations on the mechanism of uptake of myo-epoxide in *S. pombe* CBS.

It has been reported (23) that although isomytilitol (2) itself is a growth inhibitor of *S. pombe* CBS, like myo-inositol, it also antagonizes upto a certain extent, the growth inhibiting and cytotoxic effects of myo-epoxide. It has been also reported (25) that both, the myo-inositol (1) and the isomytilitol (2) enter *S. pombe* CBS through an active transport system; isomytilitol being a competitive inhibitor for the entry of the natural growth factor. Since myo-inositol diminished the uptake of myo-epoxide (Table 5 and Fig 4), and since it is known that a competition occurs between myo-inositol and isomytilitol for their entry in the cells, it was logical to examine if isomytilitol also prevented the uptake of myo-epoxide.

Fig 6 shows the uptake of (^{14}C) myo-epoxide by *S. pombe* CBS in presence of isomytilitol, 2,4-dinitrophenol (DNP), and control, for a period of 150 min. For 20 min, isomytilitol did not inversely affect the uptake of myo-epoxide. The maximum uptake, in all cases, was found to reach in 20 min. After 20 min, and upto a period of 150 min, the radioactivity uptaken by cells in presence of isomytilitol was less by 6.5 to 17 % as compared with the control. The uptake-inhibition was more pronounced in the presence of DNP, which however followed a similar pattern (Fig 6). From 20 min till 150 min, about 45 % less myo-epoxide was taken up in comparison with the control.

In another experiment, *S. pombe* CBS cells (39 mg/ml incubation mixture) from log phase were incubated with (^{14}C) myo-epoxide (61 250 CPM/ ml) and with or without glucose (1 %) at 30° for 105 min. After the incubation, the cells were harvested, washed and the uptaken radioactivity

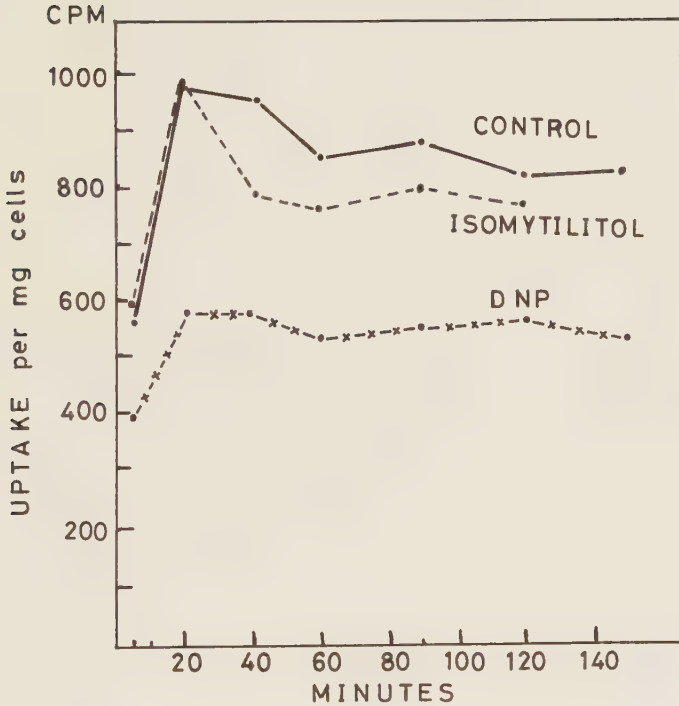


Fig 6. Inhibition of myo-epoxide uptake in S. pombe CBS by isomytilitol and 2,4-dinitrophenol.

29.6 mg cells from log phase (24 h) were suspended in 2.6 ml of 0.1 M acetate buffer (pH 5.0) which additionally contained 0.2 % glucose, 10^{-4} M dithiothreitol and (14 C) myo-epoxide (187 660 CPM = 246 μ g). The test mixtures also contained either 33.3 μ g isomytilitol/ml or 2,4-DNP (1.5×10^{-3} M). After the incubation period, the cells were rapidly centrifuged, washed and counted in Bray-Cab-O-Sil mixture.

was counted by the usual procedure. The results are reported in Table 8. The influence of glucose on the uptake of myo-epoxide by the cells is evident. The uptake was 2.5 -fold more in presence of glucose than in its absence.

Table 8

Effect of glucose on the uptake of (^{14}C) myo-epoxide by S. pombe CBS for a period of 105 min. Values are reported per ml of the incubation mixture.

Glucose in the incubation mixture, as %	(^{14}C) <u>myo</u> -epoxide, CPM		Percent uptake
	Offered	Uptaken	
1	61 250	2 775	4.5
0	61 250	1 050	1.7

6. Irreversibility of myo-epoxide action.

S. pombe CBS cells from log phase were harvested, washed and suspended in 2 ml Pennington medium without myo-inositol (54×10^7 cells/ 2 ml). The cell suspensions were then incubated with increasing amounts of myo-epoxide, as shown in Table 9, at 30° for 3 h with intermittent shaking. After the incubation period, the treated cells were harvested by centrifugation and washed free of any superficial myo-epoxide. They were resuspended in water and an uniform volume (9×10^6 cells) from each treatment tube was inoculated in 10 ml normal Pennington medium which now contained myo-inositol as usual (1 μg / ml). Growth was measured after further incubation at 30° for 44 h. All

operations were carried out under sterile conditions.

In none of the treatments, which ranged from 0.26 mM to 31.25 mM myo-epoxide, the treated cells fully recovered during 44 h growth period. Clearly, the damage to the cells increased with increasing dose of myo-epoxide. Thus, a concentration of 2 mg myo-epoxide per 2 ml treatment mixture (= 5.20 mM) killed all of the treated cells (about 54×10^7), which resulted in no subsequent growth in the normal medium. Even at half this dose, there was little, if any, subsequent growth in the normal medium (Table 9).

Table 9

Irreversibility of myo-epoxide action in S.pombe CBS.

<u>Treatment</u> <u>myo-epoxide (mM)</u>	<u>Growth (44 h) in normal medium</u>	
	<u>Percent</u>	<u>Cell morphology</u>
0	100	Normal
0.26	76	Elongated
0.52	70	Elongated
1.56	61	Elongated
2.60	9	Elongated & deformed
5.20	(2)	No growth
7.81	0	No growth
31.25	0	No growth

7. Chemical reaction of myo-epoxide with biologically important compounds .

Table 10 summarizes the chemical reactivity of myo-epoxide towards various biologically important compounds. It is evident that myo-epoxide, like various other biological alkylating agents (46,50,56,76,96,97,101-103), readily reacts with nucleic acids, nucleotides, nucleosides and bases, amongst other classes of compounds.

Nucleic acid components.

A. Bases.

Because of the poor solubility of nucleic acid bases in water, they were dissolved in 0.005 N KOH and were allowed to react with myo-epoxide (see methods). The reaction products were separated by chromatography. To determine the extent of reaction, the unreacted bases were eluted with 2 N HCl and the UV absorbance was read at the respective wave-lengths in a spectrophotometer. Table 11 shows the Rf values of reaction products and the percent reactivity of bases towards myo-epoxide. One of the reaction products, the slow migrating one, from guanine, showed a high fluorescence in UV light. Under these experimental conditions (basic) the reactivity of these bases towards myo-epoxide was found to be in the following order:

Guanine > Adenine $\approx$ Uracil > Thymine > Cytosine					
%	66	58	57	49	41

Table 10

Chemical reactivity of myo-epoxide towards biologically important compounds.

Name of the compound	Reaction	Number of reaction products obtained.		Separating system
		Major spots	Minor spots	
1	2	3	4	5

Nucleic acid components:

Bases (in 0.005 N KOH)

Guanine	+	2	1	A-1 ; A-2
Adenine	+	2	1	A-1 ; A-2
Cytosine	+	2	-	A-1 ; A-2
Uracil	+	2	-	A-1 ; A-2
Thymine	+	2	-	A-1 ; A-2

Bases(in water)

Guanine	+	1	-	D-8
Adenine	+	2	1	C-3 ; A-3

Nucleosides

Guanosine	+	3	-	D-8 ; A-1
Adenosine	+	1	-	A-3 ; C-3
Uridine	+	1	-	A-1
Cytidine	+	2	1	A-1
Deoxyadenosine	+	1	-	A-1

Nucleotides

5' - GMP (2 Na)	+	3	1	D-8
5' - AMP (2 Na)	+	3	-	C-7 ; C-3 ; D-9
5' - UMP (2 Na)	+	1	1	C-7
5' - CMP (2 Na)	+	1	2	C-7
5' - GTP	+	1	-	A-1
GDP-Mannose	+	1	-	A-1

CONT ...

Table 10 cont.

1	2	3	4	5
<u>Nucleic acids</u>				
Calf-thymus DNA:				
Native	+	See further in text		
Heat denatured	+		"	
Yeast RNA	+		"	
<u>Amino acids</u>				
Glycine	-	0	0	A-1 ; A-13
Proline	-	0	0	A-13
Serine	-	0	0	A-13
Tyrosine (in NaOH)	-	0	0	A-13
Arginine	-	0	0	A-13
Histidine	+	3	0	A-3
Tryptophan	+	1	1	A-3
<u>Carboxylic acids</u>				
Acetic acid	-	0	0	A-1
Citric acid	+	2	1	A-1
Malic acid	-	0	0	A-1
Oxalic acid	-	0	0	A-1
<u>Vitamins; co-enzymes</u>				
Nicotinamide	+	2	0	A-1
Nicotinic acid	+	1	0	A-1
NADH	+	2	0	A-1
<u>Miscellaneous</u>				
Imidazole	+	2	0	C-3
Pyridine	+	1	0	A-1
Collidine	+	1	0	A-1
Dithiothreitol	-	0	0	A-1

Table 11

Reaction of nucleic acid bases with myo-epoxide. Reaction was carried out in 0.005 N KOH.

Base	Separating system (ascending)	Rf			Amount of base reacted; %
		Base	Product I	Product II	
Guanine	A-1	0.45	0.08 (F)*	0.17	66
	A-2	0.51	0.09 (F)	0.25	
Adenine	A-1	0.63	0.08	0.23	58
	A-2	0.66	0.17	0.33	
Uracil	A-1	0.71	0.23	0.44	57
	A-2	0.71	0.23	0.44	
Thymine	A-1	0.78	0.35	0.55	49
	A-2	0.78	0.35	0.55	
Cytosine	A-1	0.68	0.22	0.38	41
	A-2	0.82	0.11	0.26	

* Light blue; strong fluorescence in UV light.

B. Nucleosides.

All of the ribonucleosides were found to be reactive towards myo-epoxide (Table 10). Because of the poor water-solubilities of the purine nucleosides, especially that of guanosine, quantitation of their reactivities with myo-epoxide could not be attempted. Pyrimidine nucleosides were, however, examined, which showed the following order of reactivity:

	Uridine	>	Cytidine
%	68		43

The order of their reactivity was analogous to that of the bases (Table 11).

C. Ribonucleoside monophosphates.

The sodium salts of ribonucleoside monophosphates were reacted in 0.05 M phosphate buffer (pH 6.6) with myo-epoxide in equimolar ratios at 37° for 2 h. The reaction products were separated by chromatography (system, C-7). The unreacted nucleotides were eluted and their concentrations were determined by reading absorbance in a UV spectrophotometer at appropriate wave-lengths. Table 12 summarizes the results. It is interesting to remark that the order of reactivity of ribonucleotides (Table 12) as well as that of bases (Table 11) remained :

Purines > Pyrimidines

Table 12

Reactivities of ribonucleoside monophosphates towards myo-epoxide.

Ribonucleoside monophosphate	Reaction %
5' - GMP (2 Na)	60
5' - AMP (2 Na)	59
5' - CMP (2 Na)	48
5' - UMP (2 Na)	38

Nucleic Acids.

A. Ribonucleic acid.

Commercial yeast RNA was reacted with myo-epoxide as described in the methods. It was observed that through out the reaction period (27 h) the pH of the reaction mixture went on increasing. It was constantly maintained between 6.8 and 8.2 by adding dilute HCl. The reaction was stopped, though incomplete, which was obvious from a

continuation of the pH increase, by adding ethanol to make 80 %. This precipitated the RNA, which was designated as myo-epoxylated RNA or "RNA-OX".

A sample of RNA-OX was hydrolysed with N HCl at 100° for 1 h. A sample of control RNA was similarly treated. The hydrolysed samples were spotted on cellulose plates and developed twice in the system D-4. The spots were marked in UV light. The results are shown in Fig 7. The slow-migrating additional spot (Spot I) obtained from the hydrolysis of RNA-OX showed a strong fluorescence in UV light. It would be seen in the later section that acid hydrolysis of myo-epoxylated DNA also gave a similar spot, with same Rf (Fig 7). It is well known that such strong fluorescence in UV light is a characteristic property of guanine products.

In another experiment, 10 mg commercial yeast RNA was dissolved in 3 ml of 5 % NaCl solution and was incubated with (¹⁴C) myo-epoxide (128 800 CPM = 169 µg) at room temperature for 40 h. The pH change in the reaction mixture was not controlled. At the end of the reaction period, an aliquot was dialysed with constant stirring against water (5 changings) in cold room for 5 days. Even after such a prolonged dialysis, it was noticed that a significant amount of radioactivity remained attached with RNA in the non-dialysable fraction. Thus, in the last changing, the dialysis water contained only 4 CPM per ml, whereas 2 030 CPM per ml remained in the dialysis bag.

B. Deoxyribonucleic acid.

Calf-thymus DNA was reacted with myo-epoxide according to the procedure described in methods. The myo-epoxylated product, DNA-OX, was precipitated by adding ethanol to make 80 %. A sample of DNA-OX was hydrolysed with N HCl at 100° for 1 h and the products were separated



Fig 7. Reproduction of the chromatogram of N HCl hydrolysed RNA-OX and DNA-OX samples, the controls and the identification spots.
System : D-4; twice migration.

by chromatography. The results are reproduced in Fig 7. Under these conditions, purines (guanine and adenine were identified) along with some unidentified spots were liberated from both the DNA and the RNA. The slowest migrating spot (spot I) from each, the DNA-OX and the RNA-OX showed a bright fluorescence in UV light. On the basis of evidence presented with other alkylating agents (48,76,78,79), this bright fluorescent spot could correspond to a myo-epoxide - guanine reaction product.

Consideration of the foregoing results suggested that the guanine moiety in nucleic acids might be one of the targets of alkylation by myo-epoxide..If this is so, then the guanine:adenine ratio in the myo-epoxylated DNA should decrease. To test this hypothesis, DNA control and DNA-OX were hydrolysed with N HCl and the products were separated by the system A-4. The spots corresponding to guanine and adenine were eluted in 0.5 N HCl and their concentrations were determined by reading absorbance at appropriate wave-lengths (248.5 and 262.5 nm respectively). The results reported in Table 13 confirm that in the myo-epoxylated DNA the guanine:adenine ratio was decreased by 0.1 from the control values.

Table 13

Guanine:Adenine ratio in myo-epoxide treated DNA(calf-thymus).

DNA	Guanine:Adenine molar ratio
Native	0.8
Treated	0.7

Heat-denatured DNA.

A sample of calf-thymus DNA (10 mg) was dissolved in 10 ml of 5 % NaCl solution and was heated to 90° in a water bath for 30 min. It was then rapidly cooled to 0° in less than a min. This heat denatured DNA was allowed to react with myo-epoxide (50 mg) at 40° for 24 h. A sample from this reacted product, on acid hydrolysis followed by chromatography in the system A-4 (ascending) also showed the presence of additional spot I as was the case in native DNA and RNA (Fig 7).

Characterization of various reaction products.

As shown in Table 10, a wide range of biologically important compounds were reacted with myo-epoxide and the products were isolated by chromatography etc. The purification of these products posed a number of technical difficulties. Characteristic properties of some of these products were studied. Yet other studies are in progress. Besides classical elemental analysis, we also attempted to characterize these products by UV and fluorescence spectrophotometry and the NMR methods. Some of these results are presented and discussed in a later section.

8. Growth inhibitory effects of some monoepoxides on

S. pombe CBS.

Epoxides are well known as a group of cytotoxic biological alkylating agents. It was, hence, interesting to test their growth inhibiting effects on S. pombe CBS, the susceptible strain, in comparison with those of myo-epoxide. Table 14 summarizes the results.

Out of eight compounds tested, 2,3-epoxy-3-phenyl - ethyl propionate (EPEP) was found to be the most toxic (ID-50 = 0.31 mM) epoxide for this yeast. Its toxicity was found to be in the vicinity of that of the myo-epoxide (ID-54 = 0.37 mM). ID-50 for α -epichlorhydrin (EP) was 1.28 mM,

Table 14

Growth inhibition in S. pombe CBS by mono-epoxides.

FORMULA NUMBER	EPOXIDE	FORMULA	CONCENTRA- TION (mM)	GROWTH(HRS) %	CELL - MORPHOLOGY *
(5)	MO		0.37	46 (44h)	El.
(11)	EPEP		0.31	50 (15h)	S.El.
(12)	EP		1.28	50 (15h)	El., D., B.
(13)	BO		23.30	50 (15h)	S.El.
(14)	PO		14.81	50 (15h)	- -
(15)	GG		29.97	50 (15h)	S.El.
(16)	ECT		30.0	100 (15h)	N
(17)	DEN		15.3	30 (44h)	El., D., Spt.
(18)	DSNT		19.4	100 (44h)	N

* El, Elongated ; S.El., Slightly Elongated ;
N, Normal ; D, Deformed ; B, Branched ;
Spt., Septed.

about 4-fold less toxic than EPEP. All of the rest of the compounds showed very weak toxicity. It must be added, however, that some of these epoxides are volatile compounds at room temperature. Thus their amounts added in the medium (concentrations shown in Table 14) might not represent their actual concentrations in the medium at any given time.

Out of the three pentol derivatives tested, only 1,4-dioxa-6-hydroxy-7,8-epoxy-(4,4) spiro nonone (DEN) showed any growth inhibition in S. pombe CBS. 1,4-dioxa-(4,4)-spiro nonone triol- 6,7,8/O (DSNT) is not an epoxide. It was included in the test to examine whether the toxicity shown by DEN was due to the epoxide moiety or due to the remaining spiro ring (see structures : Table 14). It became clear from the results that the toxicity shown by DEN must have been from the epoxide moiety. No toxicity was shown by DSNT upto a concentration of 19.4 mM, whereas DEN inhibited the growth to an extent of 70 % at a concentration of 15.3 mM. At 27 mM of DEN a cent percent growth inhibition (not shown in the Table) in S. pombe was obtained.

9. Effects of myo-epoxide on some biochemical properties of S. pombe CBS.

In order to locate the site of action of myo-epoxide in S. pombe CBS some biochemical parameters were considered. Studies on the nature of phospholipids (15), composition of cell-walls (16) and respiration (17) in S. pombe CBS grown in presence of myo-epoxide has been published. On unit cell basis, no inhibition of cell respiration due to myo-epoxide in intact cells was reported (17). In the light of present findings, that cell-envelope in S. pombe acts as a point of permeability barrier for the uptake of myo-epoxide (Tables 3 and 6), it became self-evident to test its effects on the respiration of cellular homogenates.

A. Respiration on cellular homogenates.

Respiration experiments were performed in Warburg apparatus following the classical method (153). The results (Table 15) did not reveal any inhibitory effects of myo-epoxide on respiration in cell-homogenate from S. pombe CBS. It was, however, observed that respiration in homogenates, in all the treatments including the control, was much less (about 10 times less) than what was reported for the intact cells (17). The reason for such low values could not be detected.

B. Nucleic acid composition.

The alkylation of DNA may possibly lead to an inhibition of the DNA replication, the DNA-dependent RNA synthesis or both. It was, hence, pertinent to examine the changes, if any, in the amount of DNA and RNA in the cells produced due to an influence of myo-epoxide. Table 16 shows the nucleic acid composition of myo-epoxide -inhibited (50 %) culture in comparison with the normal one. On unit cell basis, the inhibited cells possessed 3-fold more dry mass than the normal cells. This was in agreement with the observed cell morphology, which also showed about 3-fold elongation in the average cell length of the inhibited cells. The RNA content of the elongated cells was found to be 2.97 -fold more than the normal cells. The DNA content, however, in both the normal and the elongated cells remained almost unchanged (Table 16). Thus it could be seen that in myo-epoxide inhibited cells the DNA replication was arrested while the RNA and the protein synthesis proceeded almost normally - at least quantitatively, if not qualitatively.

Table 15

Effect of myo-epoxide on the respiration of cell-homogenate from S. pombe CBS . The respiration was allowed to proceed for 90 min.

Treatments	O ₂ consumed*	CO ₂ evolved*	R.Q
	μl/mg homog.	μl/mg homog.	
Homogenate (control)	1.42 ± 0.11	1.21 ± 0.07	0.85
Homog. + glucose	1.68 ± 0.08	1.34 ± 0.12	0.79
Homog. + glucose + <u>myo</u> -epoxide	1.73 ± 0.04	1.54 ± 0.11	0.89

* The values are avg. of 3 experiments ± Average Deviation.

$$A.D. = \frac{\sum (X - \bar{X})}{n}$$

Table 16

Nucleic acid composition of S. pombe CBS. Effect of myo-epoxide inhibition.

Items	Culture	
	Control	Inhibited (50%)
Average weight in g per cell (×10 ⁻¹¹)	3.7	11.2
RNA in mg/ g cell mass	51	44
RNA in g per unit cell (× 10 ⁻¹²)	1.89	4.92
DNA in mg/ g cell mass	1.76	0.617
DNA in g per unit cell (×10 ⁻¹⁴)	6.51	6.9
Ratio RNA/DNA per unit cell	29	71

C. Osmotic Shock Extract (OSE) from *S. pombe* CBS.

Neu and Heppel (143) have reported that osmotic shock extract in *E. coli* contained most of the liberated enzymes. Similar proteins or soluble material has been reported (154,155) to be involved in the transport of galactose, galactosides and aminoacids. Such substances are thought to be loosely bound at the external site of the bacterial membrane as indicated by their ready release by the osmotic shock. The object of the following experiments was to find if the OSE contained similar enzymes in *S. pombe* CBS, and, if any of the contents of the OSE could be linked with the binding and permeation of myo-epoxide.

The procedure for obtaining OSE from the cells is described in the methods. Table 17 summarizes some of the properties of the OSE from *S. pombe* CBS. The sucrase activity was taken as an indicator of the enzymes liberated in the OSE.

It appears from the consideration of the Table 17, that the culture conditions has a marked effect on the nature of the OSE. When cells from an old culture (38-40 h) were given a shock for 10 min at 35-37°, they liberated 1.2 µg protein in the OSE per mg cells, whereas a culture in log phase (24 h growth) liberated about 27 % lesser protein when subjected to an osmotic shock for 15 min at 30°. The sucrase activity was also found to be lower (by about 36 %) in the OSE from the young cultures which were shocked at lower temperature (30°).

Uptake of (¹⁴C) myo-epoxide by the normal and the osmotically shocked *S. pombe* CBS cells.

The normal and the osmotically shocked *S. pombe* CBS cells were separately suspended in 1% glucose solution which also contained (¹⁴C) myo-epoxide. Incubation was done at 30°

with constant stirring for a period of 120 min. No significant difference between the uptake of myo-epoxide by the normal and the shocked cells was observed. Hence the results are not reported.

Table 17

Properties of osmotic shock extract from S. pombe CBS.

Items : Culture conditions, treatments etc.,	Experiment series	
	I	II
Culture taken for shock (grown in Pennington medium) :		
Volume (litres)	1	1
Age (h)	38-40	24
Dry weight (g)	1.0	0.9
Shock conditions:		
Temperature (°)	35-37	30
Duration (min)	10	15
Volume of OSE (ml)	10	15
Biochemical properties of the OSE:		
Total protein liberated (mg)	1.2	0.8
Protein liberated per mg cells (µg)	1.2	0.88
Total sucrase activity present in the OSE (units)	50	24
Sucrase activity per mg secreted protein (units)	42	27

In vitro binding of (¹⁴C) myo-epoxide with the OSE.

To look for the in vitro binding, if there was any, of (¹⁴C) myo-epoxide with the OSE from S. pombe CBS the following experiments were performed. A 2 ml aliquot from the OSE (=80 µg protein) was incubated with 0.15 ml

of (^{14}C) myo-epoxide (308 600 CPM = 404 μg) at 37° for 15 min. At the end of the incubation, an aliquot was spotted on Whatman paper n $^\circ$ 1 and ascending chromatography was performed in the system 1. Two peaks from the reaction products could be revealed on the scanning. Both these peaks moved slower (Rf 0.11 and 0.34 respectively) than the reference peak of myo-epoxide (Rf 0.71). These two peaks were cut, eluted in water and re-chromatogrammed in the same system. The first peak (Rf 0.11) again remained very near the origin, whereas the second peak (Rf 0.34) now got disintegrated : 23 % of the radioactivity remained at the same spot (Rf 0.34), while 77 % of it now moved to another spot corresponding to the peak of hydroxy isomytilitol (the hydrolytic product of myo-epoxide).

It was interesting to note, that in both cases (first chromatography and the re-chromatography) UV absorbing blue spots were present corresponding to both the peaks (Rf 0.11 and Rf 0.34). It appears that always some UV absorbing material is given out in the OSE from the S. pombe cells. Similar observations have been reported in other organisms. An aliquot from the S. pombe CBS osmotic shock extract showed an absorption maxima at 265 nm.

10. Isolation and attempted characterization of (^{14}C) myo-epoxide metabolites from cellular fractions of S. pombe CBS.

S. pombe CBS was grown in normal Pennington medium (1 μg myo-inositol per ml) which also contained (^{14}C) myo-epoxide. Lots from the inhibited cultures (30-70 % inhibition) were pooled and the homogenate was prepared. The fractions were obtained as described in methods. These fractions were further analysed as reported below.

A. The 80 % Ethanol Extract.

Table 18 shows the distribution of the radioactivity from (^{14}C) myo-epoxide in various fractions. It was observed that 72 % of the total incorporated radioactivity (which in itself was very low, only about 2.9 % of the total offered in the medium; Table 4) was present in the 80 % ethanol extract fraction. Again, depending upon the yeast strain and the culture conditions employed (Table 19), 28 to 72 % of this was in the form of either free myo-epoxide or its hydrolytic product, the hydroxy isomytilitol, or both of them together. These products were identified chromatographically. The remainder of the radioactivity (72 to 28 %) was present in some bound form (Table 19 and Fig 8).

In table 20, the data of the uptake of myo-epoxide during growth (44 h) of the two strains of S. pombe and its subsequent distribution in the various peaks in the ethanol extracts from these strains, are compared. This comparison led to some interesting observations. On the basis of per mg cells, the peak A (see Fig 8) contained almost same amount of myo-epoxide (0.103 to 0.147 μg) in all the three cases. The peaks B and E were not present in the ethanol extracts from the cells protected by an excess of myo-inositol in the medium (Fig 8). Peak E, both from the resistant (132) and the susceptible (CBS) strains of S. pombe, contained almost same amount of myo-epoxide (0.05 and 0.07 μg), whereas 3.5 -fold more myo-epoxide was present in the peak B from CBS than that from the 132 strain. In the strain CBS (unprotected by myo-inositol) about 17 % of the total ethanol extract radioactivity was still available as unreacted or free myo-epoxide (0.31 μg myo-epoxide/ mg cells; peak D) after 44 h growth till the time of analysis, while in both of the remainder cases, no free myo-epoxide was left. It was further observed that 55 to 57 % of myo-epoxide from ethanol extract was present (peak C)

as its hydrolytic product (hydroxy isomytilitol) in the CBS strain, the protected one as well as the inhibited one, whereas in the strain 132 only 18 % of the ethanol extract radioactivity was found corresponding to this peak. It is interesting to consider this information in the light of the finding that much less myo-epoxide was uptaken by the protected CBS cells and that the peaks B and E were absent in its ethanol extract (Table 20 and Fig 8).

The ethanol extract from S. pombe CBS showed an absorption maxima at 260 nm. This was expected because a good deal of low molecular weight nucleic acid components from the cells would come out in this fraction.

Table 18

Distribution of (^{14}C) myo-epoxide in various fractions from inhibited S. pombe CBS cultures.

Fractions	Inhibition : 70 %		
	(^{14}C) <u>myo</u> -epoxide per mg cells		
	CPM	μg	%
Total incorporated	3 285	4.3	100
Ethanol extract	2 370	3.1	72
Water extract	250	0.33	8
Residue	641	0.84	20
From another experiment (inhibition, 40 %) the residue was further analysed)			
Alkali hydrolysable Residue			11
Alkali insoluble Residue			9

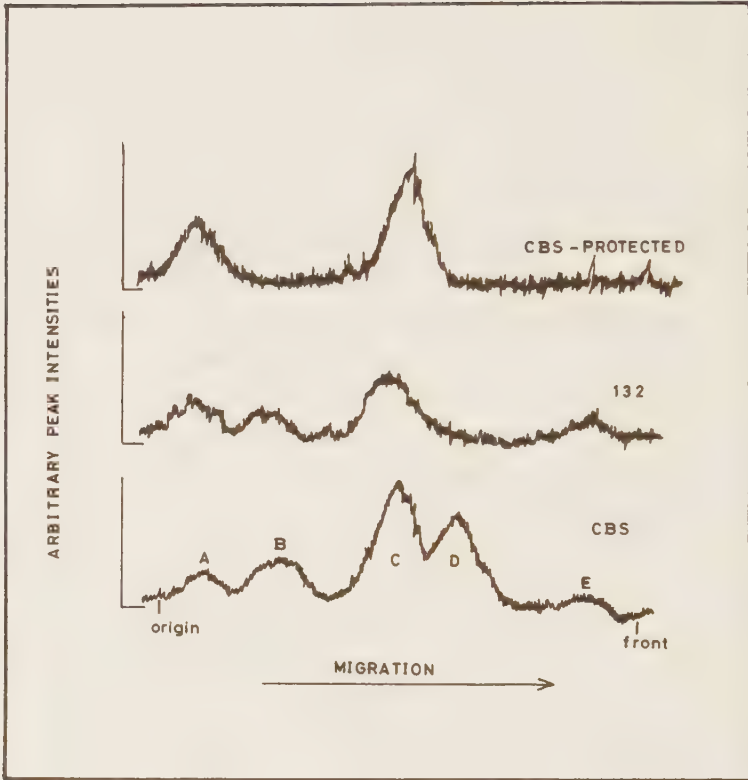


Fig 8.

Radioactivity tracings of the chromatograms of ethanol extracts from *S. pombe* strains: CBS (50 % inhibited); 132 (slightly inhibited); and CBS-PROTECTED (grown in presence of myo-epoxide but was protected by excess of myo-inositol in the medium). The peak intensities are not comparable as the scanning sensitivities were not the same in all cases.

Identification : Peak D, myo-epoxide
Peak C, hydroxy isomytilitol.

Table 19

Free and bound (^{14}C) myo-epoxide in 80 % ethanol extracts from S. pombe CBS and S. pombe 132.

Various peaks from the chromatograms (Fig 8) of the 80 % ethanol extracts were cut and the radioactivity was measured by counting. The radioactivity present in the peaks corresponding to myo-epoxide and hydroxy isomytilitol (peaks D and C respectively) were taken as the unreacted myo-epoxide and that corresponding to the remainder of the peaks was taken as the reacted or the bound myo-epoxide (see Fig 8 and Table 20).

Yeast Strain	<u>Myo</u> -inositol in the medium	(^{14}C) <u>myo</u> -epoxide present in the 80 % ethanol extract	
		Bound %	Free %
	$\mu\text{g} / \text{ml}$		
CBS	1	28	72
CBS	10	57	43
132	1	72	28

Table 20

Bound and free (^{14}C) myo-epoxide in ethanol extracts from S. pombe strains (44 h growth).
(See also Table 19 and Fig 8)

Strain	Myo-inositol in the medium ($\mu\text{g}/\text{ml}$)	Growth in mg cells per ml culture	(^{14}C) <u>myo</u> -epoxide		Peaks					
			Offered per ml medium (μg)	Uptaken per mg cells (μg)	Found in the ethanol extracts on the basis of per mg cells (μg)	Total	A	B	C*	D*
CBS	1	0.70	55	2.3	1.84	0.147	0.29	1.01	0.31	0.07
CBS	10	0.97	55	0.3	0.24	0.103	0.0	0.14	0.0	0.0
132	1	0.90	55	0.4	0.32	0.125	0.08	0.06	0.0	0.05

* Identification of peaks: D = myo-epoxide, C = hydroxy isomylitol.

The peak A from the 80 % ethanol extract.

Our attempts to identify various peaks posed complicated problems. The foremost amongst them was the unavailability of any authenticated and characterized myo-epoxide - substituted product. Many of the in vitro reaction products (Table 10) of biologically important compounds and myo-epoxide gave neighbourhood Rf values even in different separating systems. The amount of the uptaken radioactivity from the in vivo experiments was also so low that many a times a peak or a fraction contained even less than 200 CPM, when further analysis became almost impossible. Moreover, (^{14}C) myo-epoxide was not available commercially and it had to be synthesized in the laboratory. This involved certain practical problems. Thus, the labeled myo-epoxide and consequently its metabolites were in limited supply for these experiments.

Within these limitations attempts were made, however, to identify some of the metabolites isolated from the inhibited cultures of S. pombe CBS. It was observed that the acid hydrolysis of myo-epoxylated DNA and myo-epoxylated RNA gave only one additional spot (spot I; Fig 7) on chromatography in the system D-4. Peak A from the inhibited S. pombe CBS cultures gave same migration, corresponding to this spot I, in two different systems A-1 and A-4 (Rf 0.06 and 0.08 respectively). Besides, one of the spots from guanine- myo-epoxide reaction mixture also gave same Rf values in these systems. It has been well established that in vivo the principal site of alkylation of a DNA molecule by different alkylating agents is the N-7 atom of guanine (48,75,87,94,98,103,108,110,49,50). The alkylated guanine renders the N-glycosyl bond easily labile even at room temperature and at physiological pH (78,79). The removal of alkylated guanine leaves depurinated sites on the DNA molecule (48,79,81,85,87). The alkylated guanines in the

cellular pool may, therefore, possibly come from either the depurination of alkylated nucleic acids or from the direct interaction of alkylating agent with guanine compounds in the cytoplasmic pool. Consideration of these facts and the results reported above tempts us to suggest that the peak A, corresponded to the N-7 myo-epoxyl-guanine. Evidently this needs further confirmation.

B. The water extract.

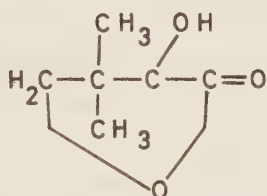
The water extract (see methods) from myo-epoxide inhibited S. pombe CBS cultures showed an absorption maxima at 255 nm and its UV absorption curve was comparable with that of a sample of commercial RNA (Fluka). This fraction (Table 18) contained 8 % of the total radioactivity incorporated by the cells. This corresponded to about 250 CPM (= 0.3 μ g) per mg cell mass. A sample from this fraction did not move from the origin on chromatography in the system A-1. Another sample, when treated with 1 N KOH at 30° for 12 h and then subjected to paper electrophoresis (system G-12) did not show any hydrolysis, while under similar treatments a sample of commercial RNA got hydrolysed giving 4 separate spots on electrophoresis. Further analysis of this fraction could not be continued because of the very low amount of counts present in it.

C. The Residue.

Table 18 shows that about 20 % of the total uptaken radioactivity was present in this fraction. From another set of experiments, the residue was subjected to hydrolysis with 1 N KOH at 30° for 12 h. The radioactivity was found to be distributed in a ratio of 11:9 between the two sub-fractions obtained, namely, the Alkali hydrolysable Residue and the Alkali insoluble Residue. No further analysis could, likewise, be continued because of the presence of too low radioactivity.

11. Experiments with pantooyl lactone.

It has been shown that pantooyl lactone (19) antago-
nized cell-division-inhibition induced by UV irradiation
in Erwinia sp. (156). It was also reported that filamenta-
tion of E. coli B, following mustard treatment could be
suppressed by pantooyl lactone (see 39). We tested this com-
pound to see if it could reverse the cell-division-inhibi-
tion and filament formation in S. pombe CBS caused by the
action of myo-epoxide.



(19) Pantooyl lactone

Two sets of experiments were performed. In one,
0.006 M to 0.1 M of pantooyl lactone was added in the growth
medium along with 80 µg of myo-epoxide per ml, and the gro-
wth was measured after 44 h. In another experiment, elonga-
ted cells (18 h growth) obtained from myo-epoxide treatment
were inoculated in 0.025 to 0.1 M pantooyl lactone contain-
ing normal medium and the growth was measured after another
40 h. The cell morphology was controlled under a phase
contrast microscope. In these cases, neither any protection
of the division-inhibition, nor any reversal of the filame-
nt formation was observed. Thus, pantooyl lactone did not
protect the growth-inhibition in S. pombe CBS induced by
myo-epoxide.

12. Inactivation of phages and phage-DNA by myo-epoxide.

A. Inactivation of E. coli phage λ .

In preliminary experiments, a suspension of phage λ particles were treated in vitro with varying amounts of myo-epoxide (Table 21). These treated phage particles were inoculated with E. coli and the plaque forming ability was measured. A treatment of 0.4 to 1 600 μg myo-epoxide per ml of 6×10^8 phage particles did not show any consistent inactivation of phages as measured by PFU (Table 21).

Table 21

In vitro inactivation of phage λ by myo-epoxide treatment.

Treatment per ml		Results	
Phage particles ($\times 10^{-8}$)	<u>Myo</u> -epoxide (μg)	PFU	Inactivation %
6	0.0	128	0
6	0.4	115	10.2
6	1.6	146	?
6	6.0	116	9.4
6	25.0	113	11.7
6	100.0	99	22.7
6	400.0	109	14.8
6	1600.0	118	7.8

B. Inactivation of DNA from the phage λ .

DNA "A" from phage λ was reacted in vitro with myo-epoxide at 37° for 60 min. The treated DNA was tested for its transforming ability in E. coli 921. A treatment of 10^{10} DNA particles with 0.4 mg myo-epoxide per ml was found to inactivate about 50 % of them and at a still higher concentration of myo-epoxide (16 mg/ml) a 100 % inactivation of DNA transforming ability was observed (Table 22).

Table 22

Inactivation of DNA from phage λ by myo-epoxide treatment.

Treatment per ml		Results	
DNA particles ($\times 10^{-9}$)	<u>Myo</u> -epoxide (mg)	PFU	Inactivation %
10	0	217	0
10	0.4	108	50.2
10	16.0	0	100

C. Inactivation of single stranded DNA phage "fd".

The single stranded DNA phage "fd" was similarly treated with 16 mg/ml of myo-epoxide and the PFU were counted. At this concentration, 98 % inactivation of these phages was observed (Table 23).

Table 23

Inactivation of single stranded DNA phage "fd" by myo-epoxide

Treatment per ml		Results	
Phage particles (10^{-9})	<u>Myo</u> - epoxide (mg)	PFU	Inactivation %
30	0	574	0
30	16	10	98

In another experiment, 14×10^9 particles of phage "fd" were treated with 0.75 to 12 mg myo-epoxide per ml. The results are reported in Fig 9. In some of the tests, along with myo-epoxide, 7.5 to 48 mg myo-inositol per ml of the treatment mixture was also added to see if there

was any protection offered by the latter. No such protective effect was observed (Fig 9). On the contrary, rather, an increase in the inactivation was observed due to the presence of myo-inositol in the treatment mixture. This unexpected synergistic effect of myo-inositol with myo-epoxide could have been due to its high concentration in the medium imbalancing the osmotic properties of the solution.

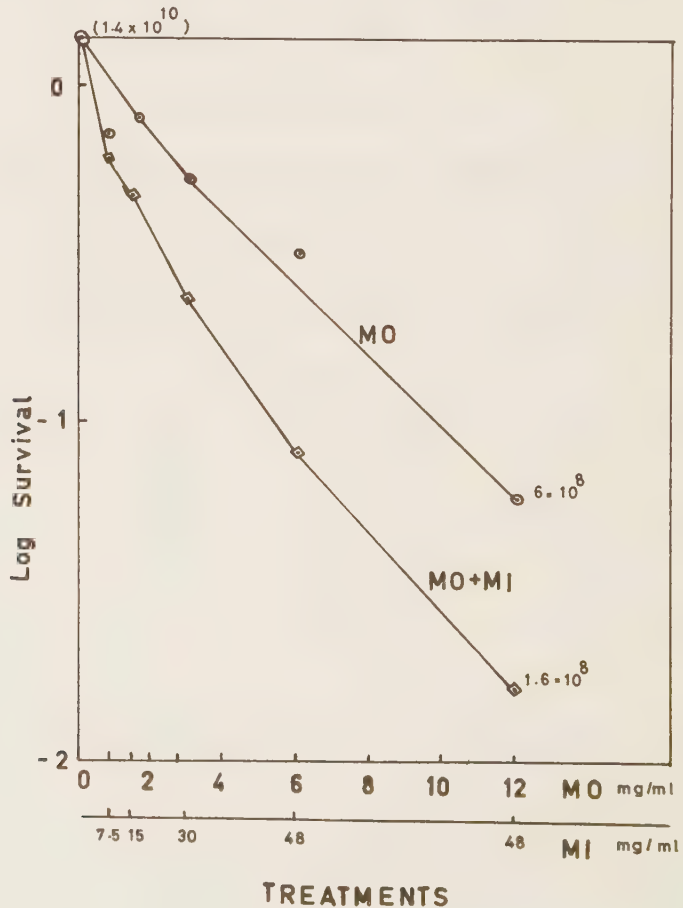


Fig 9. Inactivation of single stranded DNA phage "fd" by myo-epoxide.

13. Separation and characterization of myo-epoxide reaction products with purine ribonucleoside monophosphates.

The observation that purines were more reactive than pyrimidines towards myo-epoxide led us to examine in greater detail the reaction products of myo-epoxide and the purine ribonucleotides.

Reaction of myo-epoxide with the sodium salt of 5'-GMP.

Sodium salt of 5'-GMP was reacted with myo-epoxide in equimolar ratio at 30° for 48 h. The products were separated by two different methods, on TLC (system D-8) and by eluting through Sephadex G-25 column (system F-11).

On TLC (system D-8), 4 spots separated out, which were designated as, G-I (Rf 0.53), G-II (Rf 0.46), G-III (Rf 0.39) and G-IV (Rf 0.30). The spot G-I showed a very bright fluorescence under the UV light. Though the Rf value of the product G-II was coincident with a reference spot of GMP, its UV absorption curve was not found to be similar to that of a GMP sample (Fig 11). The spot G-II, therefore, might have been a mixture of products.

In another method (system F-11), the reaction products were separated through a Sephadex G-25 column (46.9 cm x 4 cm Ø) by eluting with 0.85 % NaCl solution. Using blue dextran, the void volume of the column was found to be 131.4 ml. The flow rate was regulated at 0.61 ml per min. Each fraction contained 3.7 ml. The fractions were collected at room temperature, using an automatic fraction collector, which was monitored by a LKB UVICORD II and a recorder unit. A typical separation is shown in Fig 10. Four peaks were separated, one of which was identified as unreacted GMP (Fig 10). The other three peaks were designated as G-1, G-2, and G-3. The peak G-1 has a shoulder in its beginning. To have minimum possible contamination, the first few fractions corresponding to this shoulder were discarded. Fractions

corresponding to the peak G-1 were pooled together and evaporated. The product was redissolved in water and passed through another Sephadex G-25 column and was eluted simply by water to obtain a desalted sample. On chromatography in the system D-8, an aliquot from G-1 was found to correspond with the product G-IV (R_f 0.30). In this system, the product G-1 gave only one principal spot plus, perhaps, another very faint and doubtful spot. Hence, the product G-1 was

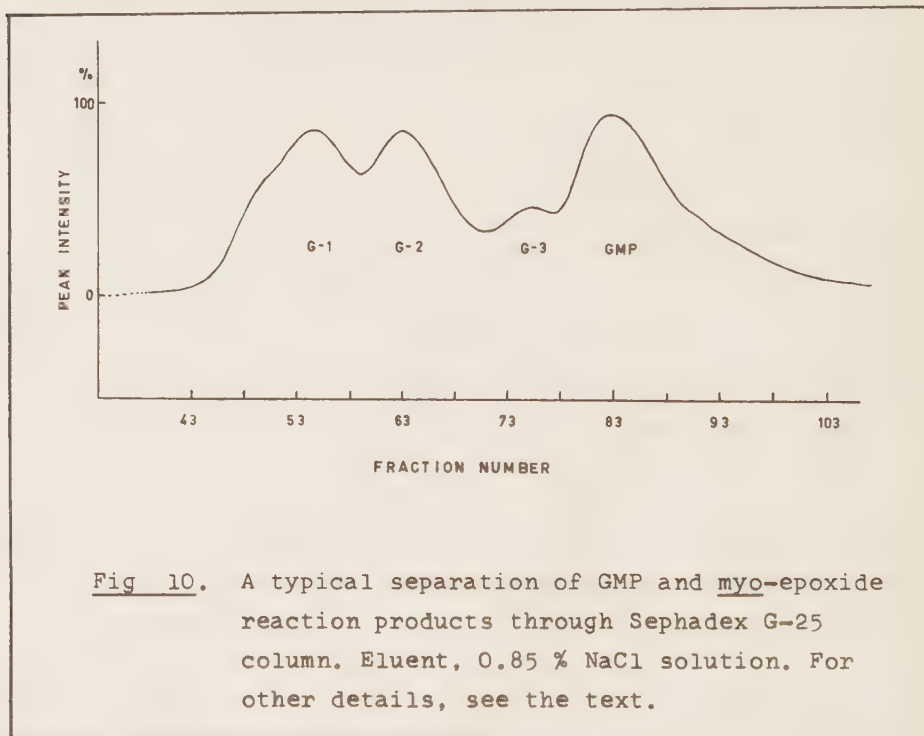


Fig 10. A typical separation of GMP and myo-epoxide reaction products through Sephadex G-25 column. Eluent, 0.85 % NaCl solution. For other details, see the text.

taken as a reasonably pure product for further studies. Not only its absorption maxima was found to change, but also the shape of its UV absorption curve was altogether different from the parent compound, GMP (Fig 11). The products G-I to

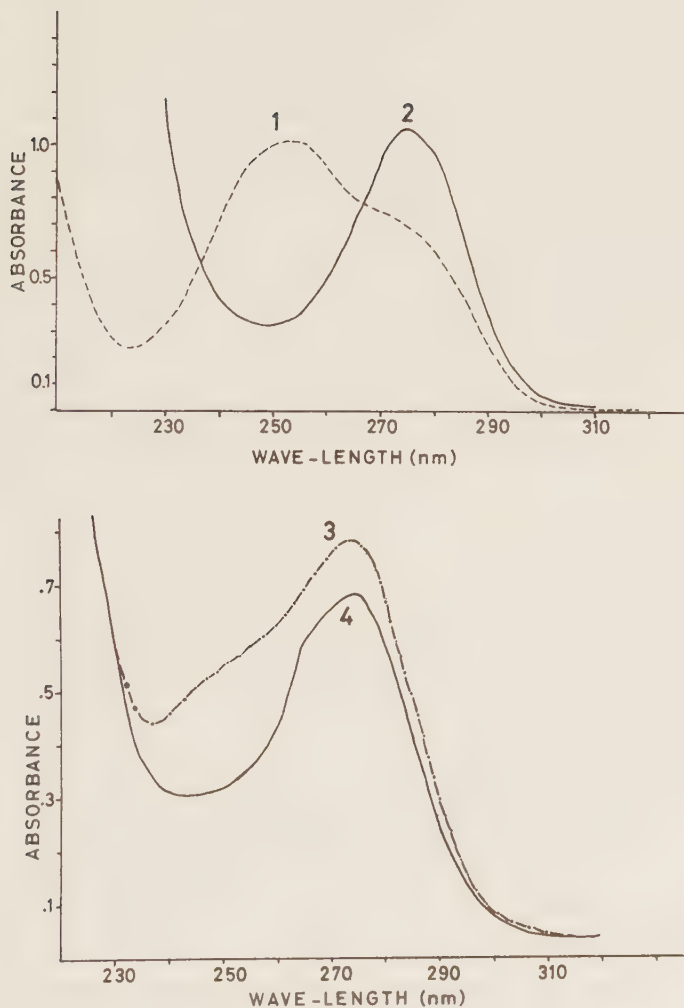


Fig. 11. Ultra-violet absorption spectra of 5'-GMP and GMP - *myo*-epoxide reaction products. The spectra were recorded in water. 1 : 5'-GMP (2Na), 2 : product G-I , 3 : product G-II , 4 : product G-I. (For further details see text).

G-IV and G-1 , all showed the presence of phosphate group when tested with the Hanes' molybdate spray on the TLC.

The elemental analysis of the product G-1 showed the following composition : C, 35.90 %; H, 4.53 %; N, 8.63 %; P, 3.8 %. This shows a C:N ratio of 4.9 . Theoretically, a mono-(myo-epoxide) substituted product of GMP will have a C:N ratio of 3.4, while a di-(myo-epoxide) substituted product of GMP will have the value of this ratio as 4.8. Thus, the product G-1 seems to be a di- (myo-epoxide) substituted GMP. Exactly on what atoms of the GMP molecule the substitution took place could not be determined.

Reaction of myo-epoxide with the sodium salt of 5'-AMP.

The sodium salt of 5'-AMP was reacted with myo-epoxide in equimolar ratio at 30° for 48 h. The products were separated by chromatography (system C-7, in cold room). Three major spots were separated which were designated as, A-1 (Rf 0.1), A-2 (Rf 0.32) and A-3 (Rf 0.89). The spot A-2 corresponded with a reference spot of 5'-AMP (2 Na). The spot A-1 was eluted and fine silica gel particles were removed by centrifugation. Checking with another chromatographic system, that is, C-3, the product A-1 gave a single spot and hence it was taken as a pure product.

Fig 12 shows the UV spectra of 5'-AMP in comparison with its reaction product A-1. It can be seen that the absorption maxima of the product A-1 has shifted to 267 nm from 260 nm that of the AMP.

Since this reaction product was not obtained in sufficient amount, full elemental analysis of the sample could not be performed. The product was, however, analysed for its phosphorus content which was found to be 3.1 %.

Fig 13 shows the fluorescence (emission and excitation) spectra of AMP and its product A-1 both in water. Tables 24 and 25 summarizes their salient features.

It should be noted that two purities of the sample A-1 were used for recording the fluorescence spectra : A-1a contained some fine silica gel particles as impurity, while the sample A-1 was practically free from any contamination. Contamination due to the silica particles in A-1a increased

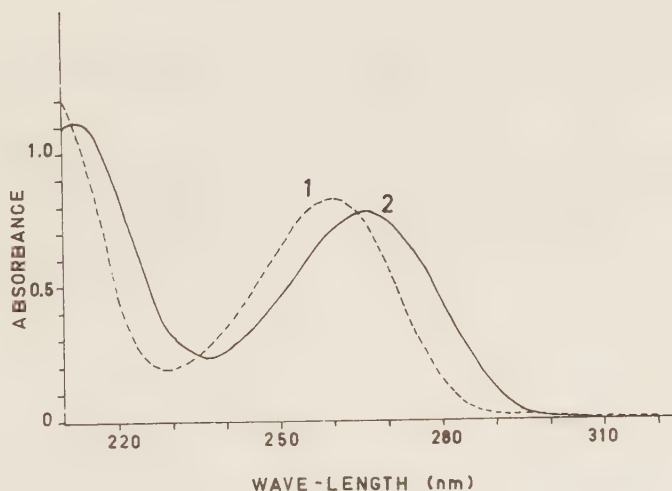


Fig 12. Ultra-violet absorption spectra of 5'-AMP (2 Na) = 1, and its reaction product with myo-epoxide, A-1, = 2. The spectra were recorded in water.

the intensity of the excitation peak appearing at 282 nm (Fig 13 C). An increase in H^+ ion concentration was also found to increase the intensity of the peak at 280 nm in the excitation spectra from both, the AMP and the product A-1 (Table 24). Similarly, an increase in the pH also affected the intensity of the peak at 280 nm.

The emission spectra (excitation at 305 nm and at 280 nm) of the compounds, AMP and A-1 were found to be

essentially similar (Table 25 and Fig 13 A and B). The emission peaks appearing at 610 nm (excitation, 305 nm) and at 570 nm (excitation, 280 nm) are not shown in the Figure, but the data have been included in Table 25. Contimation with silica gel particles gave an additional emission peak at 490 nm.

Table 24

Summary of Fluorescence-Excitation spectra (emission at 340 nm).

Compound or product	in Water		in Acid, pH $\approx$ 1		in Alkali pH $\approx$ 13	
	wave-length (nm)		wave-length(nm)		wave-length(nm)	
	Peaks at	maximum intensity at	Peaks at	maximum intensity at	Peaks at	maximum intensity at
5'-AMP(2 Na)	305	305	305	305	305	305
	280		280	280*	280	
	245		235 [§]		235 [§]	
A-1a	305		305		305	305
	280	280	280	280	282	282*
	240		240 [§]		235	
A-1	305	305	305	305	305	305
	282		282		282	
	235 [§]		235 [§]		235	

* The intensity of peak at 280/282 nm was either equal to or slightly less than that of the peak at 305 nm.

§ Presence of a shoulder.

Table 25

Summary of Fluorescence-Emission spectra.

(a) Excitation fixed at 305 nm:

Compound	in water emission peak at(nm)	in acid (pH 1) emission peak at (nm)	in alkali (pH 13) emission peak at (nm)
5'-AMP	340 380 [§] 610	340 380 [§] 610	340 380 610
5'-AMP + silica parti- cles	340 380 [§] 490 610	- - - -	- - - -
A-1	340 380 [§] 610	340 375 [§] 610	340 380 [§] 610

(b) Excitation fixed at 280 nm:

A-1	315 570	- -	- -
A-1 plus silica gel particles	310 490 565	- - -	- - -

§ only a shoulder.

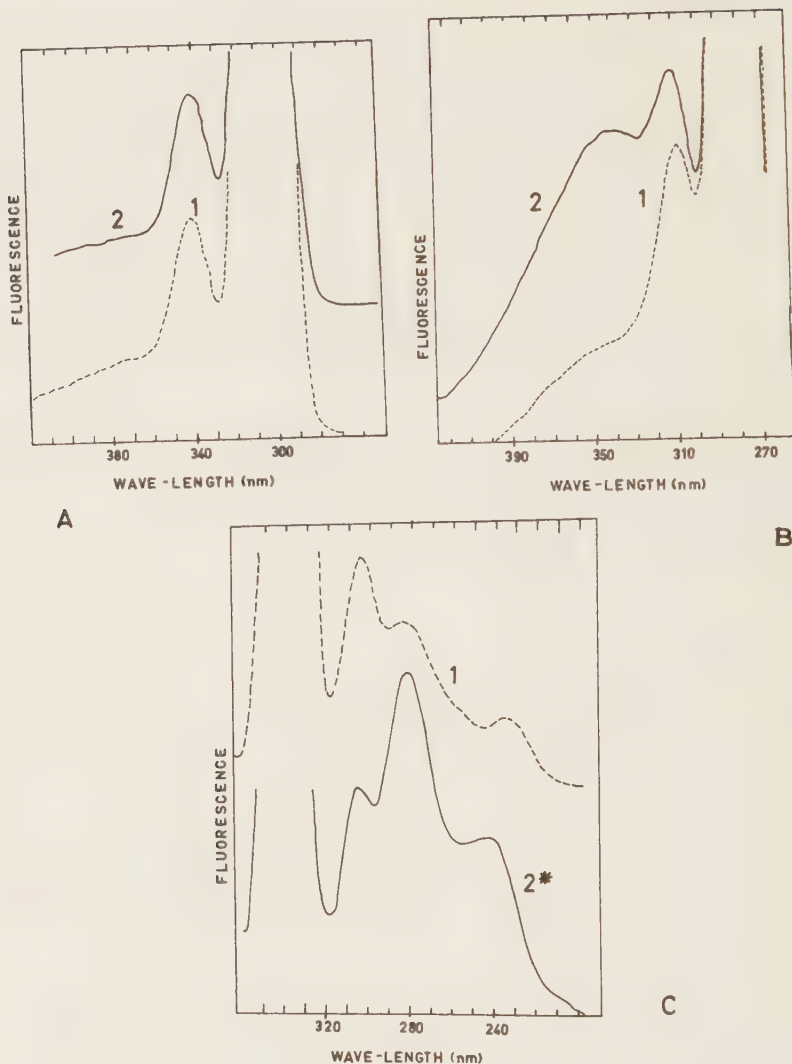


Fig 13. Fluorescence (Emission and Excitation) spectra of 5'-AMP (2 Na) and the product A-1 in water. A and B, Emission spectra when excitation was fixed at 305 nm and 280 nm respectively. C, Excitation spectra when emission fixed at 340 nm. 1 = AMP, 2 = the product A-1, 2* = product A-1a.

NMR study of the reaction products.

The NMR spectra were recorded on either Varian 60 Mc/sec or a Perkin-Elmer 90 Mc/sec instrument. These spectra were recorded in D_2O taking sodium tetra deuterium -2,2,3,3-(trimethyl silyl)-3-propionate as reference. As usual, the reactions of myo-epoxide and purine nucleosides or nucleoside monophosphates were performed in equimolar ratios.

From a GMP-myo-epoxide reaction mixture at 35° after 24 h, the peak (δ = 3.03 ppm) due to methylene of myo-epoxide was found to be displaced at a δ value around 3.6 ppm. This shift was also verified by comparing with the spectrum of hydroxy isomylitol under similar conditions. In a control sample containing only myo-epoxide, the peak at 3.03 ppm was still present (though slightly diminished in intensity) after 48 h; but was found to be totally shifted to 3.6 ppm after 12 days. After a period of 24 h reaction the peak corresponding to H-8 of the guanine ring (δ = 8.18 ppm) was found to be markedly diminished in its intensity from the GMP-myo-epoxide reaction mixture. The displacement of $-CH_2-$ peak from myo-epoxide along with the retrenchment of the intensity of H-8 peak from GMP could be explained as follows. Myo-epoxide attacks N-7 of the guanine moiety. In the resulting compound, the energy of the H-8 proton changes so that it can be easily exchanged with the deuterium of the solvent; the net effect being a decrease in the H-8 peak intensity. With adenosine, similar observations were made (Fig 14).

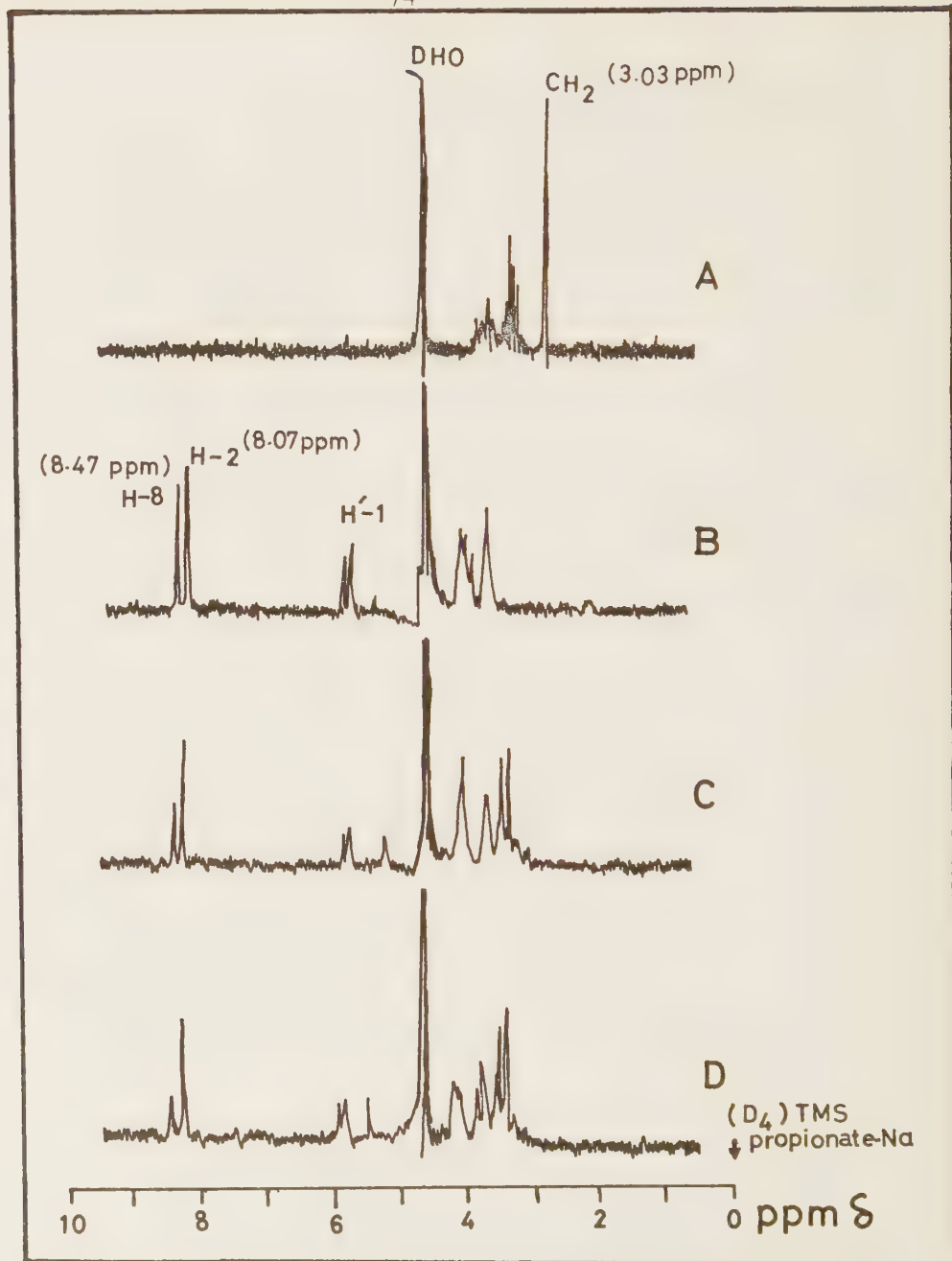


Fig 14. NMR spectra (in D_2O) of: A, *myo*-epoxide and B, adenosine. C and D, reaction mixtures of *myo*-epoxide and adenosine after 12 h and 48 h respectively.

DISCUSSION

The principal objective for this investigation was to ascertain the molecular basis for the cytotoxic, caryotoxic and growth inhibition mechanisms of the action of myo-epoxide (5) in Schizosaccharomyces pombe. The working hypothesis was adopted that myo-epoxide, much in resemblance with several other epoxides, acts by alkylating the most critical cellular target, the DNA. It was pointed out that an apparent paradox existed when the protective action (23) of myo-inositol against the inhibitory action of myo-epoxide in S. pombe is considered. It looked highly improbable that myo-inositol competes with myo-epoxide for a reaction site on DNA molecule.

Regardless of the ultimate mechanism by which a cytotoxic agent reacts with the cellular receptors, the former must, usually, be able to penetrate through the cell envelope barriers. It should then be able to survive competing alternate pathways inside the cell and also endure metabolic attack before finally arriving, in adequate amounts, at the site of action. Here its interaction with the receptor molecules produces what is termed as the biologic response. One of the ways through which cells resists the action of biological alkylating agents (127,128, 130,135,136,138) and various other drugs (132,134) is known to be due to the decreased uptake of the agent from the medium. Data for the uptake of myo-epoxide by various yeasts are analysed in the following paragraphs with this context.

1. Uptake of myo-epoxide : the basis of natural resistance
in yeasts and the basis of protection offered by myo-
inositol.

For the purpose of these experiments the following four yeast strains possessing different biochemical characteristics (Fig 3) were examined to interrelate the toxicity of myo-epoxide with its uptake:

- | | | |
|-------------------------------|---|--|
| <u>S. pombe</u> CBS | : | auxotrophic for <u>myo-inositol</u> ,
susceptible to <u>myo-epoxide</u> . |
| <u>S. pombe</u> 132 | : | auxotrophic for <u>myo-inositol</u> ,
resistant to <u>myo-epoxide</u> . |
| <u>S. carlsbergensis</u> 9080 | : | prototrophic for <u>myo-inositol</u> ,
resistant to <u>myo-epoxide</u> . |
| <u>S. cerevisiae</u> 2357 | : | prototrophic for <u>myo-inositol</u> ,
resistant to <u>myo-epoxide</u> . |

During their growth, the susceptible strain took up 6 to 8 -fold more myo-epoxide than any of the resistant strains (Table 3). This is in agreement with a number of other studies which showed the permeability differences as a mechanism of resistance to nitrogen mustard in various tumor cell lines (135,136,140). The decreased uptake of actinomycin has been similarly shown to account for its resistance in some microorganisms.(132,134). No data on the uptake studies of any epoxide are available for comparison.

It has been reported that nitrogen mustard inhibits the in vitro incorporation of (¹⁴C) orotic acid into the nucleic acids of a sensitive ascites hepatoma cells (AH-130) to a greater extent than it does so for the naturally resistant AH-7974 cells (140). The differences in these effects upon the two cell lines disappear when cell homogenates rather than the intact cells are used (140).

Likewise, in the present experiments no difference in the amount of binding of (^{14}C) myo-epoxide to the cell homogenates from the susceptible and the resistant yeast strains was observed (Table 6). The chromatographic patterns of the 80 % ethanol extracts from these homogenates and myo-epoxide reaction mixtures did not show either, any evidence of a qualitative difference in the reaction products (peaks A, B, and E; Fig 5). The distribution of radioactivity in other fractions (the Water extract and the Residue) also showed similar pattern in the susceptible and the resistant yeast homogenates (Table 7). This is in agreement with Wheeler and Alexander (149) who reported that the distribution of radioactivity into the sub-cellular fractions of sensitive and resistant plasmacytomas after the administration of three different (^{14}C) labeled alkylating agents was similar. On the basis of these results and the available supporting evidence from other authors, the natural resistance to myo-epoxide action in the yeasts can be attributed to the presence of a surface barrier which retards or hinders the penetration of myo-epoxide into the cells.

The permeability difference is not the only mechanism through which the cells are known to resist drug action. Thus, Harrap and Hill (137) did not observe any difference in the uptake of alkylating agents (busulphan, melphalan, and chlorambucil) by the sensitive and the resistant strains of Yoshida ascites sarcoma cells. They, on the other hand, unexpectedly found that the hydrolysis of one of the agents- chlorambucil - in the resistant cells occurred at a rate comparable to that of an aqueous solution of the drug, while in the sensitive cells it was 2.5 times slower. This would result in the maintenance of a higher concentration of chlorambucil in the sensitive cells and might possibly contribute to its selective lethal action.

The possibility of a higher myo-epoxide degrading ability of the resistant yeast strains over the susceptible one in the present experiment was also considered. It can not be, however, supported because, when (^{14}C) myo-epoxide was incubated with various homogenates and subsequently the 80 % ethanol extracts were chromatographed, the amount of the hydrolytic product of myo-epoxide, that is, hydroxy isomytilitol (Peak C) when expressed as a percent of myo-epoxide plus hydroxy isomytilitol (peaks C + D) did not differ significantly (varied from 30 to 40%) in any of the treatments (Fig 5). Moreover, in the growth experiments also, hydroxy isomytilitol (peak C in Fig 8) represented about 43 % of the total myo-epoxide taken up by the susceptible strain CBS, whereas in the resistant strain 132, it represented only 15 % of the total uptake (Table 20). Thus, a detoxification process involving a higher hydrolysis rate in the resistant strain can be ruled out.

In parallel with the mechanism of natural resistance in the yeast, the protective action of myo-inositol in the susceptible strain S. pombe CBS, can also be linked with a decrease in the uptake of myo-epoxide (Fig 4 and Table 5). A ten -fold increase in the optimal concentration of myo-inositol in the medium was found to reduce the uptake of myo-epoxide in the susceptible cells during growth, by about 9 -fold (Table 5). It induced a simultaneous recovery of hundred percent growth in the culture. Furthermore, myo -inositol was not found to protect, in any way, the quantitative (Tables 6 and 7) or the qualitative (Fig 5) binding of myo-epoxide to the homogenates from the susceptible strain. Thus, evidently, myo-inositol protection is due to a decrease in the uptake of myo-epoxide by the cells. Once myo-epoxide has entered the cells, no protection from myo-inositol is any more possible.

Myo-inositol and isomytilitol are known to enter S. pombe CBS through an active transport system (25). The apparent V values of both cyclitols are reported to be very close (25). The transport of myo-inositol in presence of myo-epoxide was also examined by same authors. They found a very low inhibition of myo-inositol entry when a high concentration of the epoxide was used (personal communication). In the present inquiry, it was considered whether myo-epoxide also enters through the same carrier system as does the other two cyclitols. Experiments of myo-epoxide uptake were performed in S. pombe CBS using similar techniques. The uptake of myo-epoxide was found to be only slightly inhibited (6.5 to 17 %) by the presence of isomytilitol in the medium (Fig 6) and that too after 20 min. The conditions of the present experiment were different from those of the myo-inositol uptake in the sense that the cells used were from the late log phase of growth. The earlier authors have used the stationary phase cells. They have also performed uptake studies for relatively shorter intervals. This may probably account for the differences in the first 20 min of our experiment, where no inhibition was observed. It is equally possible that a carrier system with two sites on it may be operating for these cyclitols.

Due to a limited supply of labeled myo-epoxide similar short duration experiments in presence of myo - inositol could not be attempted. However, on the basis of the decrease produced in the uptake of myo-epoxide during growth of S. pombe by the presence of myo-inositol (Fig 4 and Table 5) and on the basis of close proximity of V values of isomytilitol and myo-inositol, the latter can be expected to act in a manner similar to isomytilitol.

The uptake of myo-epoxide appears to be energy dependent. In the absence of glucose in the medium, the cells took up 2.5 -fold less myo-epoxide in comparison with the

control (Table 8). Similarly, 2,4-dinitrophenol inhibited (about 45 %) its uptake (Fig 6). It is generally believed that DNP uncouples the oxidative phosphorylation in the electron transport chain, meaning thereby that it prevents the ATP formation but permits the oxidation to proceed even at accelerated rate with the generation of heat. This energy, however, is not utilizable for physiological processes.

The osmotic shock extract from S. pombe CBS (Table 17) contained some unidentified material which were shown to bind with (^{14}C) myo-epoxide in vitro (see p 52). Two binding products were obtained, one of which (Rf 0.34) got disintegrated (77 %) to release hydroxy isomytilitol. Binding proteins and such other soluble material are reported to be released in osmotic shock extracts and are known to form complexes which might get dissociated depending on various conditions (143,155). In line with the results from various authors (143,154,155) who worked with bacterial systems, enzymatic activity was found to be released in the osmotic shock extract from S. pombe CBS (Table 17). Thus, 27 to 42 units of sucrase activity per mg of the secreted protein was present in the OSE (Table 17). However, no significant difference in the uptake of myo-epoxide by the normal and the shocked S. pombe CBS cells was observed (see p 51). One of the explanations could be that the system responsible for myo-epoxide entrance was not destroyed by the osmotic shock. Various low molecular weight nucleic acid components and even nucleic acid molecules themselves are known to be released in OSE. Such UV absorbing material were observed in present experiments (see p 53). It is therefore probable that the interaction of myo-epoxide and the OSE merely represented an in vitro reaction of the former with some of the nucleic acid components released in the OSE.

Attention must be drawn to the fact that even in the susceptible strain S. pombe CBS, a very low amount (1.4 to 2.9 %) of the offered myo-epoxide entered the cells (Table 4) during 44 h growth. Increasing the concentration of myo-epoxide in the medium was found to proportionately increase the uptaken amount. Furthermore, the 80 % ethanol extract from thoroughly washed cells which were previously grown on myo-epoxide containing medium, showed the presence of myo-epoxide peak (Fig 8 and Table 20). This means that free myo-epoxide was present inside the cells. Thus the probability that myo-epoxide only remains on the cell surface and does not penetrate through the cell can be ruled out.

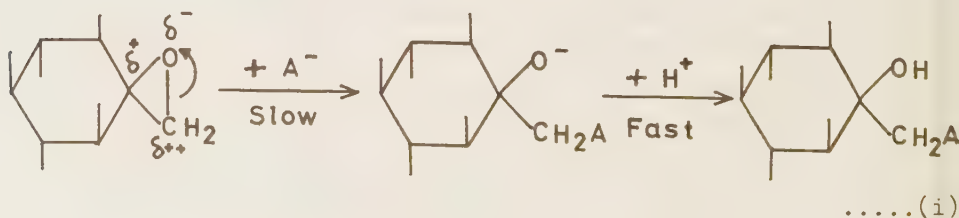
In the growth medium at 30°, about 60 % of the myo-epoxide was still present, after 44 h, in the unhydrolysed form (see p 28). Although the exact half-life of myo-epoxide was not determined, this result may be taken as an indicative of its half-life in water. In contrast with the fast reacting conventional biological alkylating agents, like various mustards, the epoxides, in general, are slow reacting agents. For example, the half-reaction time for di-2-chloroethylsulphide (sulphur mustard) molecules in water at 37° is only 3 min, whereas the half-life of diepoxybutane is reported to be about 100 h (46). Needless to emphasize that to be an effective alkylating agent under biological conditions, the myo-epoxide molecules must be transported to their site of action as neutral molecules. Looking to the biological response of myo-epoxide in S. pombe CBS and other organisms (bacteriophages etc.) this can be contemplated to be true.

2. Reaction of myo-epoxide with biologically important compounds.

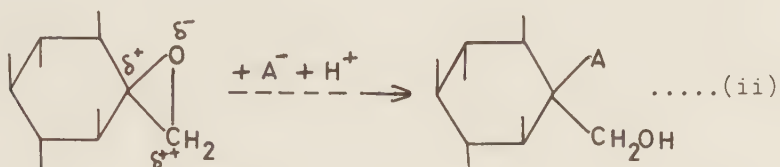
Myo-epoxide reacted with a number of biologically important compounds in vitro (Table 10). These included nucleic acid components, some amino acids, co-enzymes, vitamins and other miscellaneous compounds. Strikingly, all but one compounds with which it reacted contain one or more N-heterocycles. The exceptional compound with which myo-epoxide reacted and which does not contain an N-heterocycle is citric acid. No reaction of myo-epoxide could be detected with acetic, maleic and oxalic acids. It readily reacted with the basic amino acids, histidine and tryptophan, but no reaction could be detected with some other amino acids, like arginine and proline. Indeed, this presented some confusing picture and the mechanism of its chemical reaction needs further elucidation.

Nevertheless, myo-epoxide readily reacted with nucleic acids (Fig 7), nucleotides (Table 12), nucleosides (Table 10) and the pyrimidine and purine bases (Tables 10 and 11). Attempts were made to separate, purify and characterize some of these reaction products. Their characteristic properties have been described and discussed earlier in the text (see p 65).

In a neutral solution, the possible mechanism of myo-epoxide reaction with a nucleophilic centre (A^-) can be visualized as follows :



Because of the differences in the distribution of charge density on the exo-cyclic carbon atom and on the C-2 atom of the cycle, the epoxide ring is expected to open in the manner shown in the sequence (i). For same reasons, the sequence (ii) shown below, is not likely to be operative in the present conditions.



UNLIKELY (see text)

The first (i) mechanism is a typical SN-2 substitution. For propylene oxide, similarly, SN-2 type of reaction mechanism has been proposed (76). The rate of reaction, of course, will depend on the concentration of the reacting centres.

The relative reactivities of nucleic acid bases and nucleoside monophosphates towards myo-epoxide showed that purines are comparatively more reactive than pyrimidines (Table 11 and 12). Guanine was found to be the most reactive of all. An extensive literature is available which supports the finding that guanine is the most reactive of nucleic acid bases towards a variety of biological alkylating agents (46-48,74,87,94,110,111). The order of the reactivity of monophospho nucleosides towards myo-epoxide in water (Table 12) is in agreement (102) with the behaviour of the conventional alkylating agents, which attack the more basic nucleotides preferentially :



In the case of nucleosides, however, the order of reactivity in pyrimidines towards myo-epoxide was found to be reversed (uridine > cytidine). The bases (in 0.005 N KOH) reacted similarly (Table 11).

Yeast RNA and calf-thymus DNA (commercial), both reacted with myo-epoxide in vitro (Fig 7). Heat denatured DNA also readily reacted (see p 46). When RNA was reacted with myo-epoxide in an unbuffered medium a continuous increase in pH was observed through out the reaction period (see p 42). Reaction of an epoxide with anions or tertiary amines (possible nucleophilic centres in RNA and DNA) in aqueous medium is expected to increase the basicity. The reaction with DNA was, however, made in a buffer at pH 5.9.

On acid hydrolysis, the myo-epoxylated DNA and the myo-epoxylated RNA, each yielded only one additional product (spot I; Fig 7) in comparison with the controls. This product showed a brilliant light blue fluorescence in UV light. Only guanine substituted compounds from nucleic acid bases are expected to give such a characteristic fluorescence in the UV light. (46,103,105).

Numerous reports are available which show that N-7 atom of guanine is the most reactive site in nucleic acid molecules, especially, the DNA, towards diverse alkylating agents (48,87,94,104,111-113). N-7 alkylated guanine is the principal alkylation product, if not the only product, obtained when such alkylated samples from in vivo experiments are acid hydrolysed (48,79,78). It is tempting to speculate that the spot I (Fig 7) obtained after acid hydrolysis of myo-epoxylated DNA and myo-epoxylated RNA is 7-N- myo-epoxide (substituted) guanine. It was also shown that the guanine:adenine molar ratio in the myo-epoxylated DNA decreases to 0.7 from 0.8 of the control (Table 13). This further supports the hypothesis that spot I is a guanine product, and that the guanine moiety in DNA molecule is the principal target of attack by myo-epoxide.

3. Molecular basis for the cytotoxic effects of myo-epoxide.

The biological response of myo-epoxide in S. pombe is manifested in the induction of nuclear division inhibition, the prevention of septum formation, and the production of elongated cells (Fig 1). In bacteria and other cells, the filament formation or cell elongation has been recognised to be a consequence of nuclear division inhibition. (39-41,60). It is pertinent to point out that under the influence of myo-epoxide, the elongated S. pombe CBS possess only one nucleus each. The inhibition of DNA replication by the mustard gas has been shown to produce elongation in HeLa cells (51). The data presented in Table 16 show that on a unit cell basis, the DNA content of the elongated S. pombe CBS cells was about the same as that of the normal cells. These results indicate that somewhere in the process of inhibition by myo-epoxide, a block is introduced in DNA replication.

The normal shape of S. pombe cell is that of a cylinder with a diameter of about 5 μm , which remains constant through out the cell cycle. The cell grows in length to about 16-20 μm , when mitosis, septum formation and the cell fission intervene to separate the two daughter cells (66). The influence of DNA synthesis, mitosis and the cell plate formation on each other is not known. However, recent ultra-structure studies have revealed that normally mitosis and nuclear division precede the initiation of the septum formation in this yeast (35,36). Since the S. pombe cells inhibited by myo-epoxide remain uninucleate, it can reasonably well be deduced that the primary target of its action is on the nuclear division process, that is on the mitotic apparatus rather than on the process of septum formation.

A perusal of Table 16 further indicates that on the

unit cell basis, though the DNA content of normal and the elongated cells remained analogous, the RNA and the protein contents of the elongated cells, increased about 3-fold each of the control values. This was consistent with nearly a 3-fold increase in the cell length. In other words, the RNA and the protein syntheses proceeded normally (at least quantitatively, if not qualitatively) in the inhibited cells. Similar results have been reported in HeLa cells, where mustard gas treatment inhibited only DNA synthesis but the RNA and the protein syntheses proceeded unaffected (51).

On the basis of semi-conservative replication of the Watson-Crick model of double stranded DNA, an inhibition of DNA replication can be attributed at various stages in the process. It can be due to a block on the DNA template itself or a modification of monomers before their incorporation in the DNA or an inhibition of enzymes associated with the DNA synthesis.

Biological alkylating agents, in general, are known to be poor enzyme inhibitors (47,49). DNA polymerase and thymidine kinase, two of the various enzymes associated with DNA synthesis, have been reported to be unaffected by mustards (51). In our experiments (not reported), myo-epoxide did not show any inhibition of glucose oxidase and peroxidase. Neither it was found to adversely affect the respiration (glucose oxidation) of cell homogenate from S. pombe CBS (Table 15). The elongated S. pombe cells formed by the action of myo-epoxide show an analogous respiration to normal cells on a unit cell basis (17). Another epoxide, namely, butadiene dioxide, also has shown no inhibition of the respiration rate in Ehrlich ascites cells (80). Moreover, as the RNA and the protein syntheses are not affected by myo-epoxide action (Table 16), it can well be deduced that the enzymes involved in these processes remain unaffected, at least for the greater part.

Consideration of these facts lends support to the first possibility, that is, myo-epoxide, like various other alkylating agents (39,45,46,48,53,54,58) reacts with the DNA molecule and renders it ineffective as a template for further DNA replication. In fact, the results discussed in the preceeding section (Tables 10-13; Fig 7) clearly show that nucleic acids and nucleic acid components are the principal nucleophilic targets for myo-epoxide attack.

On its way towards the critical cellular target (that is, DNA), a good amount of myo-epoxide is likely to react with other competing centres; and indeed, they are many in a living cell. These secondary reactions, however, are not the determining factors. For example, it may also react with RNA and nucleic acid components from the cellular pool (just to cite only two of the many possible reactive sites). But these molecular species are constantly being resynthesized during growth and their reaction with myo-epoxide is not expected to be critical for DNA synthesis or for the cell division. Of course, still another possibility is that some of the myo-epoxide substituted bases from the cellular pool may get incorporated in the DNA whereby producing a block in its subsequent replication. Various base analogs are reported to function in this manner (82). In the light of existing literature on the mechanism of action of biological alkylating agents (39,46,47,54), however, a direct interaction of myo-epoxide with DNA seems more probable. Further evidence about the direct inactivation of genetic material by myo-epoxide was obtained from the experiments with phage-DNA (Table 22) and the single stranded DNA phage "fd" (Table 23 and Fig 9).

Phages can be treated with potential alkylating agents in vitro. This ensures that the primary effect is on the genetic material rather than on some other cellular

machinery used for replication. Thus, the double stranded and the single stranded DNA phages (77,116-118,120-122,125) and the RNA phages (123,124,126) have been shown to be inactivated by biological alkylating agents. The double stranded DNA phages are reported to be more sensitive to alkylating agents bearing two functional groups (118). This is plausible on the basis of the cross-linking hypothesis (39,59). The mono-functional agents are needed in larger doses for producing analogous inactivation (120). It is argued that the act of alkylation by monofunctional agents in itself is not sufficient for inactivating the phages. Alkylated purines are known to be easily removed from DNA even at ordinary room temperature and at physiological pH. It is this depurination process, producing breaks in the DNA molecule, which is claimed to be responsible for the inactivation of phages by the monofunctional agents(120).

In vitro treatment of double stranded DNA phage λ by myo-epoxide produced, rather, a confusing picture (Table 21). The inactivation varied inconsistently and the dose-survival relationship could not be derived. In view of the above cited work (120) it appears that the dose of myo-epoxide employed for this experiment was not adequate. The thick protein coat and the densely packed DNA inside the phage particle could have contributed to a penetration hindrance for myo-epoxide, preventing it from arriving at its target site. A complete inhibition of the transforming ability of the DNA from the same phage, however, was achieved at a dose of 16 mg myo-epoxide per 10^{10} DNA particles (Table 22). Taking in consideration the 50 % inactivation dose (= 0.4 mg myo-epoxide), it can be calculated that 2.4×10^8 myo-epoxide molecules were required to inactivate each DNA molecule. These findings are consistent with the observation that monofunctional alkylation below certain level does not inactivate, even after allowing

time for hydrolysis of the alkylated bases from DNA (120). It seems that either a considerable amount of damage of this type is necessary or a further process is required which becomes only significant after a loss of such a number of bases (120). The inactivation of single stranded DNA phage "fd" by myo-epoxide (Table 23 and Fig 9), further supports the hypothesis that the primary target of myo - epoxide attack is DNA.

The distribution of (^{14}C) myo-epoxide in inhibited S. pombe cells is represented in Tables 18-20 and in Fig 8. It has been pointed out earlier, that even the susceptible strain CBS took up very small percentage of the offered myo-epoxide (Tables 18 and 20). Out of the total uptake, more than 70 % was present in the 80 % ethanol extract. This fraction represents low-molecular weight cell constituents. In the unprotected CBS strain, only 28 % of the ethanol extract radioactivity was present in the reacted or the "bound" form, while the rest of it was still free, either as myo-epoxide or its hydrolytic product, the hydroxy isomylitol (Table 19). In other words, it can be deduced that only a very small amount of myo-epoxide actually reaches its ultimate site of action. Unfortunately, since the absolute DNA content of S. pombe is comparatively very low (Table 16), our attempts to isolate myo-epoxylated DNA from this yeast were not successful. Moreover, with such low counts from (^{14}C) myo-epoxide which might possibly remain attached to the DNA, it would not have been feasible to detect them (see ref 54).

The peak A was found to be present in 80 % ethanol extracts from all yeasts (susceptible, protected, resistant), which were grown in presence of (^{14}C) myo-epoxide (Fig 8 and Table 20). On the basis of the evidence discussed earlier (see p 59) it can be inferred that the peak A was a myo-epoxide substituted guanine. This might have come from

either a reaction of myo-epoxide with guanine compounds present in the soluble cellular pool or a depurination of the myo-epoxylated nucleic acid molecules. Alkylated bases, especially the purines, which remain attached to the DNA molecule are known to give point mutations by the base mis-pairing process in the following replication, whereas the depurination and the phosphate triester breaks resulting from alkylation are reported to be lethal DNA alterations (48,51,58,81,82).

The presence of the peak A (guanine -myo-epoxide reaction product) in the ethanol extracts from both the protected CBS strain and the resistant 132 strain, along with its presence in the inhibited and susceptible CBS strain, supports the initial hypothesis that once inside the cell, myo-epoxide reacts similarly in all organisms and that no protection is offered by myo-inositol at the cellular site of myo-epoxide interaction. The absence of biological response (growth inhibition etc.,) in the protected CBS and the slightly inhibited 132 strains can be explained in terms of a diminutive availability of the attacking myo-epoxide molecules in these cells. Thus, even after 48 h growth, 0.31 µg of reactive myo-epoxide per mg cell mass was present in the inhibited CBS strain (Table 20). It ensured a continuous attack-potential at the target site. On the other hand, in the protected and the resistant cells, no free myo-epoxide was left for such an attack. This may further imply that both the susceptible and the resistant strains may have analogous repair possibilities for repairing the depurinated sites of the damaged DNA. In the absence of a constant supply of attacking myo-epoxide molecules, the initially damaged DNA in the resistant and the protected cells may get repaired in due course of growth, whereas the presence of a higher concentration of the inhibitor molecules in the susceptible CBS cells

ensures a continuous attack which may out-weigh the cellular repair capacity. When S. pombe CBS cells were treated with myo-epoxide for 3 h and reinoculated in the normal Pennington medium, only a slight recovery (depending on the concentration of the inhibitor used for the treatment) of the cells was observed (Table 9). Elucidation of the interrelationship of the treatment dose and the recovery of the cell damage needs further investigation. No reversion of the filament formation in S. pombe could be obtained by pantoyl lactone (see p61).

Some other epoxides (Table 14) were found to produce growth inhibition and morphological modifications in S. pombe, similar to those produced by myo-epoxide. The differences in ID-50 of these epoxides may have been due, at least in part, to the permeability differences and/or the differences in their detoxification by the cells.

The results presented in this study throw some light on the understanding of the molecular basis of the cytotoxicity due to myo-epoxide. Additionally, it brings forward some associated and intricate problems which need further elucidation. It may, thus, be interesting to investigate the mechanism of cellular repair of damaged DNA in the myo-epoxide inhibited cells. The in vitro alkylation of phages by myo-epoxide can profitably be exploited for further mutation studies. The mechanism of chemical reaction of myo-epoxide with biologically important compounds needs further study. Some epoxides have shown tumor inhibition and carcinostatic properties. Myo-inositol is an important cell constituent. An epoxide ring attached to myo-inositol molecule evidently possesses additional advantages. An investigation of potential carcinogenic and/or carcinostatic properties of myo-epoxide can be recommended with out any hesitation.

1. Mechanism of natural resistance in yeasts.

S. pombe CBS : auxotrophic for myo-inositol,
Susceptible to myo-epoxide.

S. carlsbergensis 9080: prototrophic for myo-inositol,
resistant to myo-epoxide.

S. cerevisiae 2357 : prototrophic for myo-inositol,
resistant to myo-epoxide.

During their growth, the susceptible strain took up 6 to 8 -fold more myo-epoxide than any of the resistant strains. When, however, their cell homogenates were incubated with (¹⁴C) myo-epoxide no differences in the quantitative or the qualitative binding were observed. Distribution of radioactivity in various fractions obtained from these homogenates and myo-epoxide reaction mixtures was found to be similar in all these yeasts. It is, therefore, concluded that the natural resistance in these yeasts towards the

myo-epoxide action is due to uptake differences of the inhibitor.

The uptake of myo-epoxide by even the susceptible strain was, however, found to be very low. It was only 1.4 to 2.9 % of the initially offered amount in the medium. The absolute amount of the myo-epoxide taken up per mg cell mass was found to increase with the increasing concentration of the inhibitor in the medium. It is known that increasing concentration of myo-epoxide in the medium produces increasing growth inhibition and cell elongation in the susceptible strain S. pombe CBS. Since free myo-epoxide was still present in the 80 % ethanol extract obtained from thoroughly washed cells, it can be deduced that myo-epoxide passes across the cell membrane barrier with out being modified on the epoxide ring.

2. Entry of myo-epoxide in the susceptible strain, S. pombe CBS ; and the mechanism of protection offered by myo - inositol against the action of myo-epoxide.

The uptake of myo-epoxide in S. pombe CBS was found to be an energy requiring process. Absence of glucose in the medium decreased the uptake of myo-epoxide by 2.5 -fold. Similarly, the presence of 2,4-dinitrophenol ($1.5 \times 10^{-3}M$) in the medium decreased its uptake by about 45 %.

Myo-inositol and isomytilitol are reported to enter S. pombe cells through an active transport system. We examined the system to see if the entry of myo-epoxide was inhibited by these cyclitols. During a 150 min uptake experiment, isomytilitol decreased the uptake of myo-epoxide by 6.5 to 17 %. On the other hand, during the growth of this yeast, an excess of myo-inositol (10 $\mu g/ml$ medium) in the medium reduced the uptake of myo-epoxide by more than 8-fold. Myo-inositol was not found to decrease the binding of myo-epoxide with cell homogenate constituents. Thus, it

is concluded that the mechanism of protection offered by myo-inositol against the action of myo-epoxide is only at the site of the latter's entry in the cell. Once myo-epoxide has entered the cell no further protection due to myo-inositol is possible.

3. Chemical reactivity of myo-epoxide.

Myo-epoxide reacted, in vitro, with the heat denatured and the native DNA (calf-thymus), RNA (low molecular weight), and the nucleic acid components, like, bases, nucleosides and nucleoside phosphates. On acid hydrolysis, myo-epoxy-lated DNA and myo-epoxylated RNA, each gave only one reaction product. On the basis of chromatographic and UV absorption properties, this product is tentatively identified as myo-epoxide substituted guanine. The guanine-adenine ratio in myo-epoxide reacted DNA was found to decrease to 0.7 as compared to the control value of 0.8. On the basis of these results, it is concluded that in nucleic acids the principal site of attack by myo-epoxide is the guanine moiety.

Attempts were made to purify and characterize some of the in vitro reaction products from myo-epoxide with GMP and AMP. Ultra-violet, fluorescence and NMR spectra of these products are reported and discussed.

Besides nucleic acid components, myo-epoxide also reacted with imidazole, pyridine, collidine, nicotinamide, nicotinic acid, NADH, histidine, tryptophan and citric acid. The mechanism of electrophilic reaction of myo-epoxide with nucleophilic centres is discussed.

4. Growth inhibition in S. pombe CBS by various epoxides.

The growth inhibiting properties of 7 mono-epoxides in S. pombe CBS were studied. All of them, except 1,2-epoxycyclopentone triol 3,4,5/0, were able to produce growth inhibition and cell elongation, similar to those produced by

myo-epoxide. Their ID-50 values, however, varied between 0.31 mM and 29.97 mM. 2,3-epoxy-3-phenyl ethyl propionate (ID-50 = 0.31 mM) was found to be closest to myo-epoxide in toxicity.

5. Molecular mechanism of the action of myo-epoxide.

In S. pombe CBS, on the unit cell basis, the DNA content of myo-epoxide inhibited cultures was found to be analogous to that of the normal cultures, whereas the protein and the RNA contents of the former were 3-fold higher each. In view of about 3-fold increase in length of the inhibited cells, it became evident that myo-epoxide inhibits the DNA replication in the cell, while the RNA and the protein syntheses proceed unaffected.

Myo-epoxide was not found to inhibit the respiration in cell homogenates from the S. pombe CBS.

The distribution of myo-epoxide in cellular fractions obtained from S. pombe CBS grown in presence of (¹⁴C) myo-epoxide was studied. Out of the total myo-epoxide taken up by the cells, about 70 % was present in the ethanol extract (80 %) fraction, 8 % was present in the ethanol-insoluble, water soluble fraction obtained by high speed centrifugation, and the remaining 20 % was present in the residue.

One of the (¹⁴C) myo-epoxide metabolites present in the ethanol extract was identified as myo-epoxide substituted guanine on the basis of chromatographic migration in two systems. This metabolite, designated as peak A, also corresponded with the only reaction product obtainable from acid hydrolysis of myo-epoxylated DNA. It is proposed that at least a part of this myo-epoxide substituted guanine have come from the depurination of the cellular DNA. Alkylation of guanine by myo-epoxide is likely to result in depurinated sites and breaks on the DNA molecule. This may

have been responsible for the arrest of the DNA replication in the cells. This mechanism is suggested to be the cause of nuclear division inhibition, cell elongation and other cytotoxic effects in S. pombe CBS induced by myo-epoxide.

Myo-epoxide was also found to inactivate in vitro the single stranded DNA phage "fd" and the transforming ability of the DNA from the double stranded DNA phage λ .

In the light of these results, it is concluded that the principal cellular target of myo-epoxide action is DNA, most likely the guanine moiety in the DNA molecule.

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